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STUDIES ON CALCIUM-REGULATION IN

TWO TYPES OF MUSCLE

by

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ABSTRACT

The hypothesis that plasma membrane, smooth endoplasmic reticulum, rough endoplasmic reticulum and mitochondria of the smooth muslce of rat uterus can all transport caclium in a manner compatible with a role in relaxation, and that the mechanisms of calcium transport are different in each case, was tested.

Individual fractions composed substantially of material derived from the plasma membrane (PM), smooth endoplasmic reticulum (SER), and rough endoplasmic reticulum (RER) were isolated by the use of differential and sucrose density gradient centrifugation techniques. A fraction composed substantially of mitochondria (MITO) was isolated by differential centrifugation.

The fractions were characterized by electron microscopy and the assay of enzyme markers. The fractions were all able to bind calcium in the presence of ATP and in all cases except MITO calcium uptake was enhanced by oxalate. All fractions removed calcium from a solution of $1uM$ free calcium and although MITO could not remove calcium from 0.3 or $0.03uM$ solutions, PM, SER and RER could.

The use of drugs, monovalent, divalent and trivalent

metal ions, different substrates and reduced temperature pointed to many differences between MITO and the other fractions but to none among PM, SER and RER.

Skeletal muscle of the rat treated to the same isolation procedure as the smooth muscle produced vesicles enriched in material from the plasma membrane (PM) and vesicles enriched in material from the sarcoplasmic reticulum (SR), as well as a mitochondrial fraction. Separation and purification in the case of skeletal muscle was not as good as that for the smooth muscle. SR showed most of the properties normal for a sarcoplasmic reticulum fraction, indicating that the isolation procedure did not drastically alter calcium transport mechanisms. PM could also transport calcium, suggesting that calcium transport is a property of skeletal muscle plasma membranes.
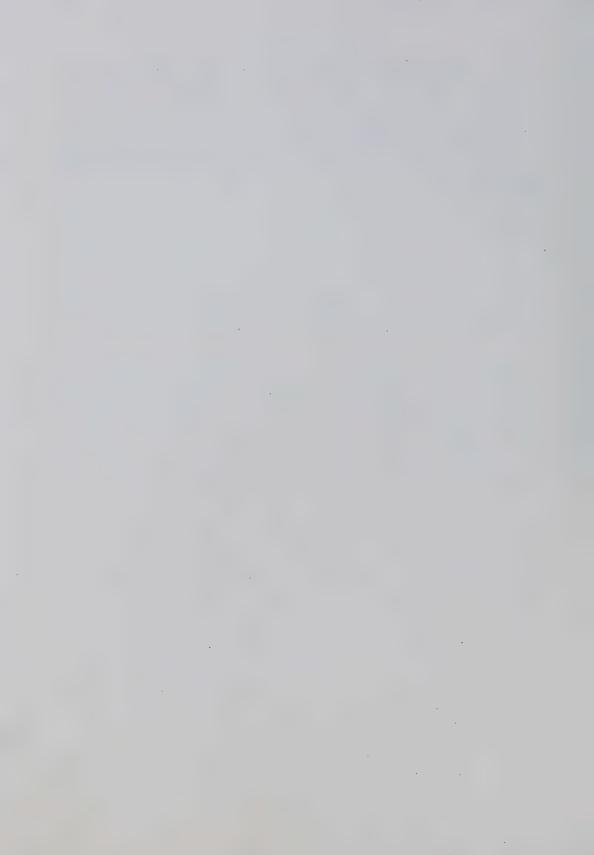
Acknowledgement.

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List of abbreviations.

AcP	acetyl phosphate.
ADP	adenosine diphosphate.
ATP	adenosine triphosphate.
ATPase	adenosine triphosphatase.
CTP	cytidine triphosphate.
E	the calcium-stimulated, magnesium-dependent adenosine triphosphatase enzyme.
EDTA	ethylenediamine tetra acetic acid.
EGTA	ethylene glycol bis (beta-amino ethyl ether)-N,N' tetra acetic acid.
EP	the phosphorylated intermediate in the reaction mechanism of the calcium-stimulated, magnesium-dependent adenosine triphosphatase.
g	gramme.
GTP	guanosine triphosphate.
ITP	inosine triphosphate.
mEq	milliequivalent.
mg	milligramme.
MgATP	magnesium adenosinetriphosphate.
MITO	mitochondria, mitochondrial fraction.
ml	millilitre.
mm	millimetre.
mM	millimolar.
mOsm	milliosmole.
nM	nanomolar.
Pi	inorganic phosphate.
PM	plasma membrane, plasma membrane fraction.
PNPP	para-nitrophenolphosphate.
RER	rough endoplasmic reticulum, rough endoplasmic reticulum fraction.
SER	smooth endoplasmic reticulum, smooth endoplasmic reticulum fraction.
SR	sarcoplasmic reticulum, sarcoplasmic reticulum fraction.
ug	microgramme.
uM	micromolar.
UTP	uridine triphosphate.

It seems to me that, by some stroke of luck, I
haven't remained ill-natured, but only disgusted
for the rest of my life with bourgeois people,
Jesuits and hypocrites of every sort.

                    Stendahl, from 'Vie de Henry Brulard'.

Chapter 1

INTRODUCTION

This thesis is concerned with the problem of
regulation of free calcium concentration in the cytoplasm
of rat skeletal and uterine muscles. I have studied rat
myometrium more extensively than rat skeletal muscle and
will deal with it first in subsequent chapters, since only
the more important experiments were repeated on skeletal
muscle. However, since the major part of our knowledge of
calcium regulation at this time comes from work on
skeletal muscle, and since inferences have been drawn from
skeletal muscle to smooth muscles, this introduction to
the subject will deal with skeletal muscle first and
smooth muscle second.

A. Calcium in muscular contraction.

1) Skeletal muscle.

The calcium ion plays a fundamental role in the
molecular events which lead to contraction of skeletal
muscle. Heilbrunn and Wiercinski (1947) were amongst the
first to suggest that calcium ion was required for the
activation of skeletal muscle; their experiments showed

that calcium is the only physiologically occurring cation that can induce shortening when microinjected into isolated frog skeletal muscle fibres. More recently Niedergerke (1955) produced similar results by electrophoretic introduction of calcium ions into frog muscle fibres and Caldwell and Walster (1963) reported similar findings after the microinjection of various substances into large leg muscle fibres from the spider crab. The dependence of muscle activation on the cytoplasmic calcium concentration has been studied in several large muscle preparations with the calcium chelating agent ethelylene glycol bis (beta-amino ethyl ether)-N,N'-tetra acetate (EGTA). Thus Portzehl, Caldwell and Ruegg (1964) showed that contractions of the giant fibres from the legs of the spider crab were initiated when the sarcoplasmic calcium concentration was raised above 0.6uM. Hagiwara and Nakajima (1966) and Ashley (1967) reported similar requirements for the production of detectable contractions in the large muscle fibres from the barnacle. The results from the experiments of Portzehl et al. mentioned above also indicated that the resting level of sarcoplasmic calcium in the spider crab fibre was probably less than 0.1uM.

Evidence to support the hypothesis that contractile stimuli lead to an increase in cytoplasmic calcium concentration, which in turn leads to contraction of

single barnacle muscle fibres, was obtained by Ashley and Ridgway (1970) using the protein aequorin which emits light in the presence of calcium. In all cases where contractions were elicited Ashley and Ridgway (1970) saw a rise in the free intra-cellular calcium concentration. Treatment with hypertonic solutions which had no effect on the membrane potential response to electrical stimulation but which abolished the normally evoked tension devolpment, also greatly reduced the calcium transient. Unfortunately, the quantitative changes in the free calcium concentration in the sarcoplasm at various times could not be accurately estimated by this technique.

Evidence for the involvement of calcium in muscular contraction has also come from studies of glycerol-extracted muscle. Muscle fibres treated with glycerol are said to retain almost all the features of the contractile systems of living muscle whilst having the ''permeability barrier'' of the plasma membrane removed (Ebashi and Endo, 1968). Bozler (1952) showed that in the presence of magnesium and ATP, very low concentrations of calcium could activate glycerol-extracted rabbit psoas muscle. Later Bozler (1954) and Watanabe (1955) showed that ethylenediaminetetra acetate (EDTA) could relax previously contracted glycerol-extracted muscle fibres even in the presence of excess magnesium ion. A different kind of preparation first described by Natori (1954), namely the

skinned frog muscle fibre which is ''nothing but living
muscle fibre minus excitable membrane'' and ''might
perhaps be the most appropriate preparation for
observation of the regulatory action of calcium ion''
(Ebashi and Endo, 1968), was used by Podolsky's group
(Podolsky and Costantin, 1964; Hellam and Podolsky, 1969;
Podolsky and Teichholz, 1970; and Ford and Podolsky,
1972a), to demonstrate that application of calcium to such
a fibre caused contraction, and that the threshold for
force development was at a calcium concentration of
approximately 0.05uM. This group also obtained results to
suggest that maximum force was developed at a calcium
concentration of 1uM and that the time course of the fall
off in force following a stimulus is essentially that of
the removal of calcium from the myofilaments.

Ashley and Ridgway (1970), however, claimed that the
free calcium concentration in the sarcoplasm of the
barnacle giant muscle fibre falls to the resting level
whilst peak tension is still maintained and suggested that
either relaxation is not dependent on the rate of removal
of calcium from the contractile machinery or that the
removal of calcium from the contractile machinery was in
some way masked to their technique.

If an increase in sarcoplasmic calcium concentration
is needed as the initiator of skeletal muscle

conctraction, then an explanation must be provided for how the sarcoplasmic calcium concentration becomes elevated as a result of a stimulus.

Hill (1949) showed that full activation of skeletal muscle occured so soon after stimulation that it could not be initiated by the diffusion of calcium ions from the cell surface, since diffusion would be far too slow. The identification of the sarcoplasmic reticulum (of which more will be said later) by Bennett and Porter (1953) and of the T-tubular systems by Porter and Palade (1957) led to explanations by which Hill's arguments could be reconciled with the evidence which indicated that calcium was the activator. The mechanisms underlying the inward spread of activation of skeletal muscle from the plasma membrane, along the transverse tubular system to the sarcoplasmic reticulum, are poorly elucidated, complex and too extensive to be dealt with here. This topic has recently been reviewed succinctly by Costantin (1971a) and more fully by Huxley (1971).

The results obtained by Costantin, Franzini-Armstrong, and Podolsky (1965) suggest that activating calcium is released from the sarcoplasmic reticulum and some support for such a mechanism comes from the results obtained by Winegrad (1968, 1970) in an autoradiographic study of calcium movements during contracture and

relaxation. As to the question of how this calcium might
be released, the present status of knowledge concerning
the link between the inward spread of activation and the
release of activating calcium has been summed up by
Costantin (1971a), who said, ''Perhaps the only valid
conclusion to be drawn at this stage is that the mechanism
of activator release is still very much an unresolved
question.'' Once calcium has been freed to the cytoplasm,
no matter by what mechanism, how does it initiate
contraction?

The exact role of calcium in the molecular events of
muscle contraction took a rather long time to be
elucidated; however, the processes are gradually being
better understood (see Tonamura and Oosawa, 1972). It is
now fairly well agreed that the fundamentally important
step in muscular contraction is the interaction of the two
contractile proteins actin and myosin in the presence of
ATP (Ebashi and Endo, 1968; and Tonamura and Oosawa,
1972). However, Weber and Winicur (1961) showed that some
preparations of actomyosin required calcium ion for
interaction and superprecipitation whilst others did not;
these observations were an extension of the observations
made much earlier by Perry and Grey (1956) as to the
effect of EDTA on the ATPase of differently prepared
actomyosins. Thus it appeared that at this level the
calcium ion may not exert any influence. The results of

Weber and Winicur also suggested that the difference in
calcium sensitivity of the actomyosin preparations
depended upon differences in the isolation of actin.
Ebashi and Ebashi (1964) were able to prepare a protein
from skeletal muscle which was different from actin and
from myosin and which was able to restore calcium
sensitivity to calcium insensitive actomyosin
preparations. This protein closely resembled that which
Bailey (1946) had first prepared some years earlier and
called tropomyosin. However, since pure tropomyosin did
not restore calcium sensitivity to calcium insensitive
actomyosin, Ebashi and Ebashi tentatively named their
protein ''native'' tropomyosin.

The explanation of the differences between pure and
native tropomyosin came when Ebashi and Kodama (1965)
separated a different, globular protein from native
tropomyosin. This protein, which they called troponin,
promoted the aggregation of tropomyosin but did not itself
confer calcium sensitivity to calcium insensitive
actomyosin systems. Ebashi and Kodama (1966) followed
these observations by showing that calcium sensitization
of actomyosin required the presence of both tropomyosin
and troponin together. The fact that troponin is the
calcium-receptive protein has now been adequately
demonstrated (Ebashi, Ebashi and Kodama, 1967; Ebashi,
Kodama and Ebashi, 1968; and Fuchs and Briggs, 1968).

Hartshorne, Theiner and Meuller (1969) and Hartshorne (1970) were able to separate troponin into two fractions; one, which they named troponin-A, conferred calcium sensitivity on a system containing the other fraction, namely troponin-B, and tropomyosin. Troponin-B itself produced an inhibition of the ATPase of actomyosin reconstituted from pure actin and pure myosin. This inhibition was unaffected by the presence or absence of calcium, and was enhanced by the addition of tropomyosin. Since Ebashi and Endo's original speculations (1968) of some direct communication in the whole muscle between troponin-tropomyosin-actin, considerable supporting evidence for this theory has been attained, and is outlined by Tonamura and Oosawa (1972). Consequently Tonamura, Watanabe and Morales (1969) questioned whether structural changes in the troponin molecule induced by calcium, which had been suggested by the results of Wakabayashi and Ebashi (1968) and later by Han and Benson (1970), were transmitted from troponin to tropomyosin to actin. Tonamura, Watanabe and Morales (1969) found that a spin label attached to tropomyosin was influenced by small changes in calcium concentration but only when troponin was present. Similarly, a spin label attached to actin was sensitive to calcium only when the intact tropomyosin-troponin system was present. Hence some structural effect and presumably a lifting of inhibition is transmitted to actin when calcium binds to troponin. Thus the

suggestions of Wakabayashi and Ebashi (1968) and of Han and Benson (1970) were verified.

The sites of interaction between myosin and actin are most likely to be at the cross bridges which exist between the thick (myosin) and the thin (actin) filaments and were first identified by Huxley (1963). Biochemical and physical data (Lowey and Cohen, 1962; Rice, 1961; and Huxley, 1963) have been used (Huxley, 1965) to identify this crossbridge as heavy meromyosin. Heavy meromyosin is a subfraction of the myosin molecule obtained by proteolytic digestion and it contains all the ATPase and actin binding properties of the parent molecule (Mihalyi and Szent-Gyorgyi, 1953). An even smaller subfragment, S-1, has been obtained from heavy meromyosin by Mueller (1965) which still retains ATPase activity and actin binding properties. The ATPase of myosin is activated by calcium ions and either unaffected or depressed by magnesium ions (Banga and Szent-Gyorgyi, 1943) but as pointed out by Perry (1965) if calcium ion were needed directly to stimulate the myosin ATPase in the intact muscle, then a free concentration of about 1mM would have to be reached for significant activation. Barron, Eisenberg and Moos (1966) showed that in the presence of actin, the myosin ATPase was inhibited in the absence of magnesium but strongly activated in its presence. Similarly Nihei (1967) found that magnesium could

maximally stimulate actomyosin ATPase. The net conclusions drawn from this work by Bowen (1971) are that in vivo the myofibrillar ATPase is activated by magnesium ion when the concentration of calcium is very low and the ionic strength is 0.05-0.15. A proposal as to how ATP hydrolysis by myosin in the presence of actin and magnesium and calcium ions leads to shortening, based on currently available evidence, has recently been made by Nakamura (1973).

To summarize this section briefly then, whatever the coupling mechanisms of extracellular to intracellular excitation might be, stimuli produce an increase in the free calcium concentration of the sarcoplasm, probably by a release of calcium from the sarcoplasmic reticulum. The resting concentration of sarcoplasmic calcium is less than 0.1uM and as this increases calcium becomes bound to the protein troponin, a conformational change is passed through troponin to tropomyosin and onto the major protein of the thin filament, actin. By this change the inhibition imposed on actin by the proteins troponin and tropomyosin under calcium-free conditions is lifted so that actin and myosin can interact together with ATP and produce shortening of the muscle. The molecular mechanisms involved in this last step have been reviewed by Tonamura and Oosawa (1972).

2) Myometrium.

As compared with the wealth of data available for skeletal muscle, the number of observations which directly implicate calcium as the activator of uterine smooth muscle is small.

Glycerol-extracted uteri were used by Briggs (1963), who showed that contractions could be obtained when ATP and magnesium were added, but not when ATP and calcium were added. Later, Briggs and Hannah (1965) presented more data from this preparation which suggested a requirement for calcium. They showed that EDTA could relax muscles made to contract by addition of 5mM ATP and 5mM magnesium. The relaxation could be reversed by the addition of calcium; however, if 15mM magnesium were used in the first step no relaxation occured with EDTA. By using EGTA they produced relaxations which were unaffected by magnesium and reversed by calcium.

A seemingly reasonable approach to the question of the involvement of calcium as activator of uterine smooth muscle is to search for the proteins which are known to be present in skeletal muscle and from the results draw analogies about the mechanisms of contraction. Proteins very similar to all the major contractile proteins of skeletal muscle have been isolated from uteri of differing

species.

Csapo (1948, 1950a,b) was able to isolate actomyosin and myosin from uterus and since his pioneering work, both actin (Needham and Williams, 1963; and Carsten, 1965) and myosin (Cohen, Lowey and Kucera, 1961; Needham and Williams, 1963; and Wachsberger and Kaldor, 1971) have been extracted and purified. The properties of the uterine proteins are in some aspects quite different from those from skeletal muscle, the most notable differences being the increased solubility of uterine myosin and the very low activation of ATPase induced by magnesium (Needham and Shoenberg, 1967). The detailed involvement of calcium and magnesium in actin-myosin-ATP interactions are not clear (see Needham and Shoenberg, 1967). Both the actin and the myosin extracted from uterus can form actomyosin when crossed with the opposite protein from skeletal muscle (Csapo, 1971). The regulatory proteins have been isolated from myometrium; tropomyosin by many groups (Sheng and Tsao, 1955; Tsao, Tan and Peng, 1956; Jaisle, 1960; Needham and Williams, 1963; and Carsten, 1968) and troponin by one (Carsten, 1971). Carsten showed that a uterine troponin-tropomyosin complex caused an inhibition of ATPase activity of desensitized rabbit skeletal muscle actomyosin in the presence of EGTA. The report suggests that though similar results were produced when uterine actomyosin was used they were not

consistently reproducible, this fact being attributed to the instability of the actomyosin ATPase.

Consequently the evidence of Briggs and Hannah discussed above suggests that calcium can activate the myometrium and that all the proteins (or similar ones) which are needed for the calcium activated process of skeletal muscle contraction are present in the myometrium. This, taken together with the evidence (Bozler, 1968; Filo, Bohr and Ruegg, 1965; and Schadler, 1967) that there is a similar calcium dependence for contraction between glycerinated skeletal and several types of smooth muscle (though myometrium was not examined in any of these cases), presents reasonably suggestive evidence that calcium is the activator in myometrium.

B. Cellular calcium regulation.

From the preceding it has become clear that the muscle cell must regulate its cytoplasmic calcium concentration quite accurately. At rest it must be kept below 0.1uM; during activation it must be increased to around 1uM and for relaxation it must be lowered back to the resting level. There are a number of known cellular processes which can serve to regulate cytoplasmic calcium and which might serve as calcium regulators in muscle. This section is dedicated to a discussion of these

processes.

    1) The sarcoplasmic reticulum.

    Undoubtedly the most widely studied cellular calcium regulating system is the sarcoplasmic reticulum of skeletal muscle.

    Marsh (1951, 1952) showed that muscle homogenates were capable of relaxing a suspension of myofibrils in the presence of ATP. The suggestion that a labile relaxing factor (Marsh factor) was present in these homogenates was proposed. Marsh also noted that the addition of calcium to the system prevented the relaxing effect of the Marsh factor.

    Kumagai, Ebashi and Takeda (1955) and Portzehl (1957) showed that the essential component of the Marsh factor was particulate in nature. Ebashi and Lipmann (1962) considered the size and shape of the vesicles of their relaxing factors to be very similar to the intact sarcoplasmic reticular network described by Porter and Palade (1957); they concluded relaxing factor to be sealed-off fragments of sarcoplasmic reticulum.

    When Hasselbach and Makinose (1961) and Ebashi and Lipmann (1962) showed that the isolated fragments of

sarcoplasmic reticulum were able to take up calcium from a medium containing ATP and magnesium, even when the free calcium concentration was lowered to 0.1uM by EGTA, the initial link between calcium accumulation and relaxing ability was established. The result of the calcium accumulation studies on the isolated preparation suggested to Weber (1966) that the sarcoplasmic reticulum in the intact muscle was responsible for lowering the calcium concentration to that needed to produce a relaxed state. Thus a particulate fraction from skeletal muscle, apparently composed of material almost totally derived from the sarcoplasmic reticulum, was shown able to accumulate calcium.

Hasselbach and Makinose (1961) showed that the addition of oxalate to a calcium uptake reaction mixture, such that the solubility product of calcium oxalate was not exceeded, resulted in a massive increase in calcium uptake with precipitates of calcium oxalate appearing in the vesicles. These results prompted Hasselbach (1964) to suggest that calcium was being transported through the membrane and into the lumen of the vesicles. Weber, Herz and Reiss (1966) proposed that after transport across the membrane, calcium becomes bound to a low affinity site on the inside surface and that an equilibrium is established between this and free intravesicular calcium; in cases where oxalate is present the free intravesicular calcium

concentration would be kept low by precipitation with oxalate so that increased net uptake could occur under these conditions. Carvalho and Leo (1967) and Carvalho (1968) think that the effect produced by oxalate is of limited value in providing information of the true state of the accumulated calcium in these structures, and that calcium bound to the membrane represents a large fraction of the total calcium accumulated when no oxalate is present. They found fragmented sarcoplasmic reticulum preparations to have a total binding capacity of 350 mEq per mg protein. These sites are available for magnesium, potassium, hydrogen and calcium ions depending on the conditions. The presence of ATP confers on these sites a much greater affinity for calcium such that this ion can then be bound from solutions of 0.1uM.

With regards to the rate of calcium uptake by fragmented sarcoplasmic reticulum, Ohnishi and Ebashi (1964) found that up to 40 micromoles of calcium per g of protein could be taken up in 30 milliseconds. The technique they used involved spectrophotometric measurements of absorbency changes in the dye murexide, which is a metallochromic indicator sensitive to the calcium concentration in the medium, and was aided by continuous flow mixing. More recently Inesi and Scarpa (1972) using a similar technique, monitored by continuous oscilloscope tracing of the changes in murexide

absorbency, showed uptake to be linear only for the first
400-600 milliseconds. The initial rate varied between 60
and 70 micromoles calcium taken up per g protein per
second.

At least three factors have been suggested to
influence the amount of calcium that can be accumulated by
fragmented sarcoplasmic reticulum in the absence of
precipitating agents. These are: the free calcium
concentration of the medium (Weber, Herz and Reiss, 1966);
the rate at which calcium effluxes from the vesicles
(Johnson and Inesi, 1969); and the intravesicular free
calcium concentration (Weber, 1971).

The mechanism by which calcium is taken up by the
isolated sarcoplasmic reticulum appears to involve a
calcium-activated, magnesium-dependent ATPase. Hasselbach
and Makinose (1962) showed that ATP hydrolysis by isolated
sarcoplasmic reticulum was low in the absence of calcium
and in the presence of magnesium. However, when calcium
was added there was an immediate large increase in the
rate of hydrolysis. Hasselbach and Makinose (1963) and
Weber, Herz and Reiss (1966) showed calcium uptake and
ATPase activities in the presence of oxalate to be tightly
coupled in a ratio of two calcium ions transported to one
ATP molecule hydrolysed. This situation held good
irrespective of the inside to outside ratio of calcium

however, when leak of accumulated calcium occurs, the ratio is altered. Several other lines of evidence have led Inesi (1972) and Yamamoto (1973) to conclude that calcium accumulation is linked to the calcium-activated, magnesium-dependent ATPase. Thus the sulphydryl group inhibitors Salyrgan and N-methylmaleimide inhibit both calcium-ATPase and calcium uptake to the same extent (Hasselbach and Makinose, 1962; Hasselbach and Seraydarian, 1966; Inesi, Goodman and Watanabe, 1967; and Martonosi and Feretos, 1964); incorporation of the terminal phosphate from ATP to form a phosphorylated intermediate in the membrane has been demonstrated and shown to occur only in the presence of calcium, and to be stimulated at calcium concentrations equal to those required to stimulate ATPase activity (Inesi, Maring, Murphy and McFarland, 1970; Makinose, 1969; and Yamamoto and Tonamura, 1968). Further the net synthesis of ATP from ADP and inorganic phosphate has been shown to occur when calcium is released from sarcoplasmic reticular membranes (Makinose and Hasselbach, 1971; and Panet and Selinger, 1972).

Not only ATP but also ITP, GTP, CTP, UTP, (Martonosi and Feretos, 1964; Hasselbach, 1964; and Makinose and The, 1965) acetyl phosphate (De Meis, 1969; and Pucell and Martonosi, 1971) and para-nitrophenyl phosphate, but not para-nitrophenyl acetate (Inesi, 1971) can support calcium

uptake. However the rates of calcium uptake and the affinities of the uptake mechanism for calcium are lower with these alternative substrates than they are with ATP as substrate.

On the basis of currently available evidence, several essentially similar models have been proposed for the mechanism of calcium transport (Inesi, 1971; Martonosi, 1972; Makinose, 1973; Meissner, 1973; and Yamamoto, 1973). The reaction scheme given below incorporates the major points from these models.

$$E + ATP + 2Ca^{++} \rightleftharpoons E.ATP.Ca_2 \qquad (1)$$

$$E.ATP.Ca_2 \qquad \rightleftharpoons EP.Ca_2 + ADP \quad (2)$$

$$EP.Ca_2 \qquad \rightleftharpoons E + Pi + 2Ca^{++}$$
$$(3)$$

Here E represents the ATPase enzyme and EP represents the phosphorylated intermediate in the overall reaction sequence. The first steps in the transport process require the binding of ATP and calcium to the ATPase. On the basis of the results described earlier, which showed that two moles of calcium were transported per mole of ATP

hydrolysed, it has been assumed that two calcium ions bind to the enzyme in company with one ATP. Kanazawa et al. (1971) have presented data from kinetic studies on the initial rate of formation of the phosphorylated intermediate with respect to calcium concentration which shows that one E binds with two calcium ions. As to the sequence of the binding steps in part (1) of the scheme, Kanazawa et al. (1971) showed this process to be random initially, but under steady-state conditions to proceede in the ordered sequence of ATP binding first followed by calcium. Yamamoto (1972) has suggested that this difference between initial and steady-state binding may result from the fact that in the former case ATP and calcium are binding to a free enzyme whereas in the latter the enzyme is already bound to ions being transported from the inside to the outside of the sarcoplasmic vesicles as counter ions to calcium; in the latter circumstance calcium can only bind after ATP has bound and displaced these counter ions.

According to Panet, Pick and Selinger (1971), part (2) of the overall reaction, the formation of EP, is greatly influenced by variation in the free calcium concentration but not by variation in the free magnesium concentration of the reaction mixture. These results were interpreted by Panet, Pick and Selinger (1971) as indicating that although magnesium is important for the

overall transport and ATPase process, it is not necessary for the formation of EP. These workers, however, did not remove bound magnesium from their sarcoplasmic reticulum preparations. In contrast to Panet, Pick and Selinger (1971), Yamamoto (1973) reported that the rate of formation of EP is significantly lower when magnesium is not added to the reaction mixture. He suggested that MgATP is the substrate for the formation of the intermediate and that the slow but finite rate of formation observed when no magnesium was added was due to the presence of contaminating magnesium in the sarcoplasmic vesicle preparations. Like Panet, Pick and Selinger (1971), Kanazawa et al. (1971) showed that formation of EP is dependent upon the presence of calcium since the formation of EP was immediatly arrested when calcium was chelated with EGTA.

Yamamoto (1973) has reported that under appropriate circumstances (i.e., in the presence of excess ADP) the following reaction can take place almost completely stoichiometrically:

$$EP + ADP \rightarrow E + ATP$$

such a reaction clearly indicates that EP is an energy-

rich phosphate compound.

Panet, Pick and Selinger (1971) showed that step (3) of the reaction, the breakdown of $EP.Ca_2$ to give E, inorganic phosphate and the release of calcium, requires the presence of magnesium. EP, which was stable in the presence of calcium and in the absence of magnesium was very quickly hydrolysed to E and inorganic phosphate upon the addition of magnesium. Similar conclusions about the necessity for magnesium in this step were drawn by Inesi et al. (1970) and by Yamamoto (1973). Since the decay in the EP ceased gradually upon the addition of EDTA to normally prepared vesicles but ceased almost immediatly upon the addition of EDTA to sarcoplasmic reticulum preparation which had been disrupted by the addition of Triton X (à detergent), Yamamoto (1973) concluded that internal magnesium is required for step (3) of the reaction.

The question arises at to where in the membrane the various stages of the reaction take place. Since calcium is transported from the external surface to the interior of the sarcoplasmic reticulum, it can be assumed that calcium binds with E on the external surface of the membrane and is released at the internal face. But at what point in the process does translocation of calcium from outside to inside take place?

Yamada, Yamamoto and Tonamura (1970) showed that the calcium initially incorporated into the sarcoplasmic membranes at low ATP concentrations can not be removed by high concentrations of EGTA. Kanazawa et al. (1971), however, showed that the formation of EP could be stopped immediately by the addition of EGTA. Thus the E.ATP.Ca$_2$ complex appears to be located at a site which can be affected by EGTA. The reverse reaction, namely the formation of ATP and E from ADP and EP, was independent of the external calcium concentration. However, this reaction was accelerated in the presence of calcium when the preparation was treated with Triton X and could be terminated by the addition of EGTA.

At pH9.2 in which state the sarcoplasmic reticulum is permeable to calcium (Duggan and Martonosi, 1970), EP decomposes slowly in the presence of EGTA and ADP (Kanazawa et al. , 1971). This slow decomposition is eventually terminated under these conditions but can be started again by the addition of excess calcium.

These observations strongly suggest that the reaction

$$E.ATP.Ca_2 \rightleftharpoons EP.Ca_2 + ADP$$

moves from left to right under the influence of external calcium and from right to left under the influence of internal calcium, which further suggests that translocation of calcium takes place simultaneously with the formation of EP.

Part (3) of the reaction mechanism is probably not as simple as shown since the inorganic phosphate appears to be released at the external vesicular surface (Yamada, Yamamoto and Tonamura, 1970). On the basis of this observation, the fact that magnesium is required for the dephosphorylation of EP and the observations of Carvalho and Leo (1967) that the sum of the equivalents of magnesium, calcium and potassium bound to SR remains constant,. Yamamoto has proposed the following mechanism:

$$EP.Ca_1 + Mg \rightleftharpoons EP.Mg + 2Ca^{++} \qquad (3a)$$

$$EP.Mg \rightleftharpoons E + Mg^{++} + Pi \qquad (3b)$$

Step (3a) would take place at the inner and (3b) at the outer membrane surface and magnesium would be transported from the inside to the outside of the vesicle as a counter ion to calcium.

The use of partially purified (Meissner, 1973) and completely purified (MacLennan, 1970; and MacLennan et al. , 1971) calcium-ATPase preparations has lent strong support to the idea that the ATPase may be the complete calcium transporting unit in the sarcoplasmic reticulum. Thus the ATPase has specific binding sites for calcium and magnesium (MacLennan, 1970) and binds ATP and calcium in the ratio of 1:2 (Meissner, 1973). It contains a protein phosphorylation site and catalyzes an ATP-ADP exchange (MacLennan, 1970; and Meissner, 1973). Furthermore the enzyme can itself form membranes under appropriate conditions and would appear to be located within the membrane across which calcium is being transported (MacLennan et al. , 1971). Also the purified ATPase has been reconstituted into a membrane together with phospholipids and this preparation is capable of supporting calcium transport (Racker, 1972).

That fraction of the transported calcium which under physiological conditions becomes bound to the sarcoplasmic reticulum is probably bound to a protein rich in glutamic and aspartic acid residues, which can bind up to 970 nmol of calcium per mg and which constitutes 7-15% of the total protein of the sarcoplasmic reticulum, (MacLennan and Wong, 1971; and Ikemoto et al. , 1972). The protein has been named calsequestrin by one group (MacLennan and Wong,

1971) and calcium-precipitable protein by the other (Ikemoto et al. , 1972). Although there is some disagreement about its molecular weight (MacLennan and Wong, 1971; and Ikemoto et al. , 1974) this parameter is in the area of 50,000 and calsequestrin and calcium-precipitable protein appear to be identical. There appear to be two types of calsequestrin with slightly differing molecular weights and amino acid compositions occuring within a single heterozygous strain of rabbits (MacLennan, 1973).

Balzer, Makinose and Hasselbach (1968) and Balzer (1972) have shown that the drugs chlorpromazine, propranolol and verapamil inhibit calcium uptake and the calcium-ATPase of isolated sarcoplasmic reticulum vesicles at concentrations between 0.1mM and 1mM.

2) The mitochondrion.

Another system with the ability to accumulate calcium is that of the mitochondrion and recently Borle (1973) has discussed a theory that mitochondria are of primary importance in the regulation of calcium metabolism in several types of cells.

Isolated mitochondria have been shown able to accumulate large quantities of calcium by energy-dependent

processes. Vasington and Murphy (1961, 1962) showed this for rat liver mitochondria incubated with a respiratory substrate, magnesium, ADP or ATP and inorganic phosphate. Even though oxidative phosphorylation did not occur simultaneously with calcium uptake under any circumstance, calcium uptake could be inhibited by 2,4-dinitrophenol. Uptake was relatively rapid, maximal loading being achieved in 5-10 minutes at 37ºC. Lehninger, Rossi and Greenawalt (1963) found that inorganic phosphate was accumulated along with calcium in the ratio of 1.67 calcium to 1 inorganic phosphate.

In a survey of the interactions between calcium ions and mitochondria from different tissues and species, Carafoli and Lehninger (1971) outlined the salient features of calcium uptake in the presence of respiratory substrate by mitochondria from all the vertebrate tissues they studied. When mitochondria were allowed to respire either in the presence or absence of phosphate, the addition of calcium caused a sudden jump in the rate of respiration and there was an uptake of calcium and a release of hydrogen ion. The presence of phosphate was required for large amounts of calcium uptake. The presence of high-affinity calcium binding sites which were independent of respiration was detected as was an endogenous store of calcium which could be mostly released by uncoupling agents. Yeast and blowfly flight muscle

mitochondria had no high-affinity binding site and showed
no ability to accumulate calcium in a respiration-
dependent manner. This observation, together with the
fact that the high-affinity binding sites show the same
cation specificity as does calcium uptake (Reynafarje and
Lehninger, 1969) and with the observations of Mela and
Chance (1969) that lanthanum ions inhibit both
respiration-linked calcium accumulation and high-affinity
calcium binding sites, have been taken by Lehninger (1970)
to indicate that the high-affinity binding sites represent
the carriers for respiration-dependent calcium uptake. A
more extended study of blowfly flight muscle mitochondria
by Carafoli et al. (1971) indicated that they could not
''actively seggregate (calcium) at the low concentrations
at which it is presumed to occur in the sarcoplasm in
vivo.''

Rossi and Lehninger (1963) and Brierly, Murer and
Bachmann (1964) showed that calcium and phosphate could be
accumulated by the hydrolysis of ATP but in the absence of
electron transport, calcium stimulated the hydrolysis of
ATP by mitochondria. This ATP-dependent calcium uptake by
mitochondria was inhibited by oligomycin although the
calcium uptake dependent on respiration first described by
Vasington and Murphy (1961, 1962) was not (DeLuca and
Engstrom, 1961). Bielawski and Lehninger (1966) found 1.9
molecules of calcium to be accumulated per molecule of ATP

hydrolyzed. Chappell, Cohn and Greville (1963) showed that the mechanism which transports calcium in rat liver mitochondria will also transport strontium and manganese.

Thus mitochondria can accumulate calcium by two mechanisms but the precise relationship between the calcium accumulation that is dependent upon respiration and that dependent upon the hydrolysis of ATP is unclear. However it does appear clear (Carafoli and Lehninger, 1971; Lehninger, 1970; and Lehninger, Carafoli and Rossi, 1967) that under conditions closely resembling those of the cytoplasm as regards presence of ATP, magnesium and respiratory substrate, calcium can be accumulated by mitochondria by both mechanisms.

With regard to the kind of cytoplasmic calcium levels that might be maintained if they were controlled by the mitochondria Drahota et al. (1965) concluded that the uptake of calcium which is dependent upon respiration, magnesium, phosphate and either ADP or ATP, switched off when the surrounding calcium concentration fell to 1-2uM, and also that there was a steady-state maintenance of the net amount of calcium accumulated such that the external calcium concentration was maintained at 1 to 2uM. ATP could also promote uptake and retention of the same load of calcium.

The calcium accumulation by mitochondria may very
well serve as a buffer against drastic increases in
calcium concentration in some cells, in such a way that
calcium is taken up and the cytoplasmic concentration is
lowered, after which small amounts of calcium can be
released and extruded from the cell by other mechanisms,
probably at the plasma membrane (see Borle, 1973).
Certainly a pulse of calcium chloride injected into the
axoplasm extruded from the squid giant axon is quickly
inactivated as indicated by the quick fall in light output
from aequorin back to the resting level (Baker, 1972).
The facts that the metabolic poisons cyanide and 2,4-
dinitrophenol caused an increase in the free calcium
concentration in intact axons from 30 to 90 minutes after
application, (the time within which ATP falls to low
levels under the conditions used, Baker and Shaw, 1965),
and that the calcium concentration fell again when the
inhibitors were removed, indicated to Baker (1972), by
analogy with similar experiments on isolated mitochondria
(Azzi and Chance, 1969), that mitochondria might be
responsible for the calcium buffering in these axons.
When oligomycin was applied some time before cyanide in
intact axons (Baker, Hodgkin and Ridgway, 1971) there was
an immediate increase in the free calcium of the axon when
cyanide was applied. The results suggest that the
mechanism of calcium buffering in the squid axon is
susceptible to the combined effect of oligomycin and

cyanide, which blocks the electron transport of respiration; this susceptibility is the same as that shown by the calcium uptake mechanism of mitochondria. If ATP were injected into axons poisoned only with cyanide the released calcium was taken up. Those poisoned with both oligomycin and cyanide were not influenced by the injection of ATP (Baker, Hodgkin and Ridgway, 1971). Baker (1972) concluded from these results that mitochondria are very likely capable of acting as intracellular calcium buffers in the squid giant axon.

The results of Drahota et al. mentioned above raise the question whether mitochondria could keep the sarcoplasmic calcium concentration low enough to maintain the relaxed state. Probably because of the remarkable properties of the sarcoplasmic reticulum which were outlined in the previous sections, little regard has been paid to the question for skeletal muscle, although Lehninger (1970) has suggested that mitochondria might supplement or even supplant the role of the sarcoplasmic reticulum in the removal of calcium from the sarcoplasm of red skeletal muscle. Lehninger's evidence comes from the work of Patriarca and Carafoli (1969) which showed a higher content of calcium and a higher specific activity of ^{45}calcium in the mitochondrial fraction than in the microsomal fraction from red muscle of rabbits killed shortly after intravenous administration of the ^{45}calcium

chloride. The redistribution of calcium that might take place upon isolation of the fractions is unclear and these experiments do not provide proof for an involvement of mitochondria. Different opions exist amongst investigators as to the role of the sarcoplasmic reticulum in relaxation of heart muscle, and there has been strong support for mitochondria as the regulators of calcium levels from beat to beat (see Kubler and Shinebourne, 1971; and Scarpa and Graziotti, 1973);. however the evidence has been inconclusive. Patriarca and Carafoli (1968) carried out similar experiments on heart muscle as on skeletal muscle (Patriarca and Carafoli, 1969). Both they and Horn, Fyhn and Haugaard (1971), who used perfused rat hearts, found the highest specific activity of ^{45}calcium in the mitochondrial fraction, after isolation, but again the effects of homogenization and isolation on calcium stored in the sarcoplasmic reticulum and in mitochondria are unknown. Haugaard, Haugaard, Lee and Horn (1969) suggest that cardiac arrest brought about by oligomycin is due to an elevated cytoplasmic concentration of calcium as a result of the inhibition of calcium transport by mitochondria. However, the fact that hearts poisoned by oligomycin come to rest in the relaxed state (Challoner, 1968; and Challoner and Steinberg, 1966) plus the fact that oligomycin does not inhibit respiration-dependent calcium transport by mitochondria (DeLuca and Engstrom, 1961), indicate that this is not a satisfactory

explanation. Martonosi (1972) has suggested that the contracture of oligomycin-poisoned hearts in response to adrenaline, theophylline and electrical stimulation (Horn, Levin and Haugaard, 1969) may be explained better by the marked lowering in creatine phosphate and ATP concentrations under these conditions rather than by an effect on mitochondria which are involved directly in calcium regulation. Chance (1965) measured calcium uptake by pigeon heart mitochondria by monitoring changes in the redox state of cytochrome-b, which is sensitive to changes in calcium concentration (Chance and Williams, 1955), and concluded that these mitochondria had a rate of uptake and affinity for calcium sufficient to permit them to regulate calcium levels affecting contraction in the heart. These measurements were, however, indirect since actual calcium accumulation was not measured and the velocity of calcium uptake at low concentrations of free calcium was calculated from data obtained at saturating calcium concentrations. Recently, Scarpa and Graziotti (1973) used the murexide spectrophotometric technique to measure the initial rates of calcium uptake from solutions of varying free calcium concentrations, by mitochondria from the hearts of several species. They found that, even from a concentration as high as 5uM, only 0.9-1.3 nanomoles of calcium per g wet heart tissue could be removed by rat heart mitochondria at 38°C in the time required for relaxation. This value of calcium uptake was two orders

of magnitude less than the amount required to be removed from the myofibrils during relaxation as calculated by other authors, (Katz, 1970; and Langer, 1973). The same observations were true for the mitochondria from guinea pig, squirrel and pigeon hearts. Frog heart mitochondria however, could take up 6 nanomoles of calcium per g wet heart tissue during the slower relaxation time. Scarpa and Graziotti concluded that the mitochondria could not be solely responsible for the required calcium regulation in any of the cardiac tissues studied.

According to Batra (1973) the mitochondria from human myometrium can bind 87.53 micromoles calcium per mg protein in five minutes from a solution of 0.087uM free calcium. This value is much higher than those reported for mitochondria from other sources and makes calcium uptake by mitochondria a mechanism to be reckoned with in the process of relaxation of the smooth muscle. No other studies of calcium regulation by mitochondria from smooth muscles have been reported.

3) The plasma membrane.

At least two types of active mechanism have been described by which cellular plasma membranes can contribute to intracellular calcium regulation, (for references see a and b below). One mechanism relies on

the pumping of calcium from the cell in the presence of magnesium and ATP, and it resembles in some cases and in some features the mechanism of the sarcoplasmic reticulum. In the other mechanism calcium is extruded from the cell in exchange for sodium which normally enters the cell by moving down a concentration gradient maintained by the sodium pump. Details of the two processes will be outlined in this section.

a) ATP-dependent calcium extrusion.

Most of our knowledge of the cellular ATP-dependent calcium extrusion process comes from studies of red blood cells, red blood cell ghosts and red blood cell membrane fragments, (Schatzmann and Vincenzi, 1969; Olson and Cazort, 1969; Cha, Shin and Lee, 1971; and Weiner and Lee, 1972). After Schatzmann's original report in, 1966, Schatzmann and Vincenzi (1969) observed that previously ATP-depleted red blood cells that had been loaded with calcium and magnesium-ATP showed an outward net transport of calcium against an electrochemical gradient. There was a simultaneous appearance of inorganic phosphate and during the initial phase of transport 1.3 moles of Pi were released per mole of calcium extruded. This transport was highly temperature-sensitive and did not take place if cells were treated similarly but not loaded with ATP. ATPase activity was stimulated by calcium from inside the

membrane only. Neither calcium transport nor the ATPase activity were sensitive to sodium, potassium, oligomycin or ouabain but in both processes calcium could be replaced by strontium. Olson and Cazort confirmed that the red cell membrane could transport calcium and strontium and showed that GTP, ITP, but neither acetyl phosphate nor pyrophosphate, could support transport of both ions. There was no exchange of magnesium for either calcium or strontium. From the results of experiments in which they found that a variety of treatments produced parallel effects both on the calcium-activated, magnesium-dependent ATPase and on calcium transport by red blood cell membrane fragments, Cha, Shin and Lee (1971) concluded calcium uptake to be intimately linked to calcium-ATPase. Weiner and Lee (1972) later confirmed that the calcium binding seen by Cha et al. was an uptake into inside-out membrane fragments, that the accumulated calcium was lost in the absence of ATP and that this loss could be slowed in the presence of lanthanum.

The work of Borle (1969a,b) on HeLa cells and of Lamb and Lindsay (1971) on L cells and L cell ghosts suggest that a similar mechanism to that in the red cell also operates in these cases.

Isolated systems believed to be mainly plasma membrane from longitudinal smooth muscle of guinea pig

ileum (Hurwitz et al. , 1972), rabbit skeletal muscle
(Sulakhe, Drummond and Ng, 1973a) and rabbit aorta (Meyer,
1973) can accumulate calcium in an ATP-dependent manner;
this property has been taken to be the manifestation of an
outwardly directed calcium pump in the muscle plasma
membrane. The work of Hurwitz et al. (1972) demonstrated
that the calcium accumulating ability of vesicles isolated
from the guinea pig ileal smooth muscle followed the
occurrence of sodium-potassium ATPase whilst it was
independent of NADH-oxidase activity in the different
fractions of microsomes collected from a sucrose-density
gradient. Only one enzyme, namely sodium-potassium ATPase
was used to follow the occurrence of the plasma membrane
through these vesicular fractions and nothing is known of
whether these fractions could accumulate calcium fast
enough and from a calcium solution of low enough
concentration to give the plasma membrane an important
role in calcium regulation in the longitudinal smooth
muscle of the guinea pig ileum. Despite the fact that
there was an increase in calcium uptake by these fractions
in the presence of oxalate, which was taken to indicate
that transport into the vesicles was occurring, no further
evidence was offered to show that this indeed represented
a calcium extrusion process by the cell membrane. This
last criticism can also be made of the work of Sulakhe,
Drummond and Ng (1973a). These workers have quite
carefully characterized the plasma membrane fraction they

obtained from rabbit skeletal muscle; however the fraction was treated by a technique, namely lithium extraction, which Repke and Katz (1969) have shown to cause severe damage to the calcium transporting ability of some membranes, hence calcium accumulation by the skeletal muscle plasma membrane fraction may have been seriously underestimated. Again the ability of these membranes to remove calcium from the sarcoplasm, in such a way that would give them a regulatory function, cannot be estimated from the results presented. The report of Meyer (1973) cannot be evaluated at this time.

Thus as Baker (1972) points out the affinity of these ATP-dependent systems for calcium is not at all clear and whether any of them could contribute to maintaining an intracellular calcium concentration of below 0.1uM is uncertain.

b) Sodium influx-dependent calcium extrusion.

The best evidence for a calcium extrusion process dependent upon the simultaneous influx of sodium comes from the study of calcium movements into and out of the squid giant axons and has been reviewed by Baker (1972). Baker, Blaustein, Hodgkin and Steinhardt (1967) and Blaustein and Hodgkin (1969) demonstrated that the efflux of injected calcium from squid giant axons was highly

temperature-sensitive, was unaffected by ouabain, was reduced by the replacement of external calcium by magnesium and was further reduced by replacing the external sodium by either lithium or choline. The results were suggestive that calcium efflux depended partially on an exchange for external calcium and partially on an exchange for external sodium.

However, in a system as seemingly simple as the squid giant axon, studies of calcium movements are complicated by binding of the ion to internal sites. Thus the poisoning of squid axons with cyanide (Rojas and Hidalgo, 1968; and Blaustein and Hodgkin, 1969), in order to evaluate the energy requirements for calcium extrusion surprisingly resulted, after a variable lag time, in a rise in calcium efflux. The rise was attributed to a release of accumulated calcium from mitochondrial stores as a result of metabolic poisoning and to an insensitivity of the calcium extrusion mechanism to metabolic poisoning. From measurements of both influx and efflux from poisoned axons Blaustein and Hodgkin concluded that there was an increase in net efflux. They also showed the properties of calcium efflux from poisoned axons to be essentialy similar to those of efflux from non-poisoned axons. However, if calcium efflux were dependent upon the sodium gradient, then the sodium gradient must have been maintained in the poisoned axons and if it was, there must

still have been sufficient ATP to drive the sodium pump
and thus perhaps a calcium pump. Blaustein and Hodgkin
(1969) make no comment on the state of the sodium gradient
in these poisoned axons but do admit that some ATP is
probably present and that an ATP-dependent calcium
extrusion process cannot be ruled out.

A reversal of the above mechanism, namely the efflux
of sodium ions dependent on an influx of calcium has been
demonstrated (Baker, Blaustein, Hodgkin and Steinhardt,
1969). When the external sodium concentration was reduced
both sodium efflux and calcium influx increased whilst
calcium efflux declined. If external sodium were replaced
by lithium, choline or sucrose then calcium influx
increased similarly. If the external sodium concentration
were greatly increased calcium influx and the calcium-
dependent sodium efflux were inhibited, probably because
sodium was better able to compete with calcium for
membrane binding sites. The process showed remarkable
similarity to the sodium-dependent calcium efflux. It was
activated by increasing the internal sodium concentration,
and both calcium-dependent sodium efflux and sodium-
dependent calcium influx changed in parallel with the
square of the internal sodium concentration; it was
unaffected by high concentrations of cardiac glycosides
and strontium could replace calcium whereas magnesium
could not and proved to be slightly inhibitory. The

results of the experiments just described (Baker, Blaustein, Hodgkin and Steinhardt, 1967 and, 1969; Blaustein and Hodgkin, 1969; and Rojas and Hidalgo, 1968) suggest that calcium flux across the membrane of the squid giant axon is partially dependent upon the trans-membrane sodium gradient.

Whether some or all of the sodium-dependent calcium efflux could take place in the absence of energy-rich compounds was not satisfactorily answered in the experiments just described because of practical problems in reducing the concentration of these compounds sufficiently, and also in interpreting the influence of calcium release from various, probably mitochondrial, stores (see Baker, 1972). However Dipolo (1973a) used the technique of Brinley and Mullins (1967) to dialize squid axons such that the ATP concentrations were lowered to 1uM. Under these circumstances the bulk of calcium efflux was dependent on external sodium and calcium concentrations suggesting the presence of some calcium efflux mechanisms that were dependent partially upon exchange for external calcium and partially upon exchange for external sodium. In these axons various ionic gradients could be manipulated by varying the perfusate as well as the bathing solution. When ATP was added to the perfusate of the axons there was a reduction in efflux, which was interpreted as being due to stimulated uptake by

the axonal mitochondria. Dipolo also presented evidence for a direct calcium releasing effect of cyanide on mitochondria which might explain some of the inconsistencies revealed by the previous use of this technique.

Baker (1972) has calculated that a sodium-calcium exchange system of the type just outlined, with 1 calcium ion being exchanged for 3 of sodium, would be sufficient to maintain the normal resting concentration of free calcium in the nerve. It should be noted that in none of the cases described above was the simultaneous involvement of an ATP-dependent calcium pump, similar to that described in the first part of this section, ever ruled out, in fact Dipolo (1973a) has hinted of evidence to suggest that such a mechanism operates.

The evidence for a sodium-dependent calcium extrusion process from tissues other than nerve is not quite so convincing. Dipolo (1973b) and Russel and Blaustein (1974) have provided evidence that a similar mechanism operates in barnacle giant muscle fibres. Whilst the evidence is consistent with the sodium-calcium exchange mechanism it is diffucult to tell how much the results obtained were influenced by the more complex intracellular binding of calcium in muscle than as compared to nerve. Certainly some of the evidence used by the protagonists of

the sodium-calcium exchange mechanism for its existence in other tissues (see Baker, 1972; and Russel and Blaustein, 1974) has been taken out of context. Thus the work of Cosmos and Harris (1961) on skeletal muscle and of Goodford (1967) on smooth muscle did not claim to demonstrate transport mechanisms and do not do so. Reuter and Seitz (1968) and Glitsch, Reuter and Scholtz (1970) studied the efflux of calcium from isolated hearts and auricles, respectively, and claim to have evidence for a sodium-calcium counter transport in heart muscle. However, heart muscle, with its complexities of extracellular space and uncertainties of intracellular binding, is not so simple a model as the squid axon and it is not at all clear that the contribution of these additional factors has been fully accounted for. Similar arguments can be used against the claims of Reuter, Blaustein and Haeusler (1973) for a sodium-calcium exchange system in arterial smooth muscle. However, unless one rejects the idea that a rise in free cytoplasmic calcium concentration is the immediate signal for contraction, then it is hard to argue that various treatments by which sodium was replaced and as a result of which contraction occured, did not indeed result in a rise in cytoplasmic free calcium. In fact several studies have indicated an interaction between sodium and calcium ions in producing contractions in smooth muscle. Thus Daniel (1964) showed that procedures which produced an inhibition

of active sodium-pumping in uterus also produced contracture and Osa (1971) showed that mouse myometrium contracted in sodium-free, normal calcium but not in sodium-free calcium-free solutions; Bauer, Goodford and Huter (1965) showed that there was an increase in tension and calcium content of the smooth muscle of guinea pig taenia coli when external sodium was replaced by lithium; and Sitrin and Bohr (1971) demonstrated some complex interactions between sodium and calcium in producing contractions in vascular smooth muscle. Nevertheless, the results could be explained by an influence of sodium on the membrane binding of or permeability to calcium, as well as by a sodium-calcium counter transport mechanism.

In summary then the plasma membrane of the squid giant axon can regulate the axoplasmic calcium concentration by exchanging calcium ions in return for sodium ions, the energy for this process being gained from the downhill movement of sodium ions. Similar processes have been claimed to be operative in other tissues but as yet the evidence for these is incomplete.

C. Calcium regulation in skeletal muscle.

As was outlined in section B above it has generally been accepted that the sarcoplasmic reticulum of skeletal muscle is capable of accumulating and releasing all the

calcium necessary for contraction and relaxation, though the mitochondria have been suggested to contribute in some cases. But are intracellular ''sinks'' enough?

As Bianchi (1968) and Baker (1972) pointed out, the Donnan equilibrium predicts that if the ionized calcium inside cells were determined passively calcium should be accumulated by most cells. Clearly skeletal muscle, with a free calcium concentration in the sarcoplasm of 0.1uM or less, is not accumulating calcium. Unless the plasma membrane is completely impermeable to calcium, which does not appear to be the case (Gilbert and Fenn, 1957; Cosmos and Harris, 1961; and Russel and Blaustein , 1974), skeletal muscle must extrude calcium or its ''sinks'' would overflow. That a calcium extrusion mechanism is necessary in skeletal muscle cells even at rest was not thought of here but was indeed suggested by Gilbert and Fenn in, 1957 and later reiterated by Weber (1966) and by Bianchi (1968). One hypothesis (Frank, 1958) for the mechanism by which excitation is linked to the contraction of muscle fibres invokes the influx of small amounts of ''trigger'' calcium as the link. Since this hypothesis is currently receiving support from diverse types of experiments (for references see later) and since ''trigger'' calcium would eventually have to be removed from the cell, it will be dealt with in more detail.

Frank (1958) suggested that the influx of calcium played an intermediate role in the contraction of skeletal muscle since the contraction produced by high potassium was abolished by brief pre-treatment with calcium-free solution. Later, Frank (1960) showed that, although the response to calcium was quickly lost in calcium-free solutions, a contraction to caffeine, which has since been shown to release calcium directly from the sarcoplasmic reticulum (Endo, Tanaka and Ogawa, 1970), was still attainable under these circumstances. The rate of loss of response to high potassium was similar to the rate at which calcium ions left the extracellular space. Edman and Grieve (1961), Jenden and Reger (1963) and Luttgau (1963) obtained similar results but attributed them to the fall in the resting membrane potential which occurs in calcium-free media suggesting that this would block the coupling of depolarization to the release of activator. However, Frank (1964) showed that the contractile response occured before the fall in resting membrane potential. These observations were supported by those of Curtis (1963) who showed that reduction of the external calcium concentration to about 100uM produced no significant decrease in the resting membrane potential, despite the fact that the contractile response to high potassium was abolished. As a result of an investigation of the kinetics of the activation process using voltage-clamped muscle fibres, Adrian, Chandler and Hodgkin (1969) noted

that the response to activation appeared to be a regenerative process. One year later, two almost simultaneous reports appeared (Endo, Tanaka and Ogawa, 1970; and Ford and Podolsky, 1970) which indicated that a regenerative mechanism of calcium release by calcium took place in skinned skeletal muscle fibres. The experiments showed that the application of small amounts of calcium to fibres which had been allowed to take up calcium produced a faster developing and greater force of contraction than did the same application to fibres which had not been preloaded with calcium. Based on some of the experimental results just described Costantin (1971b) reiterated the hypothesis of Bianchi and Bolton (1967) that depolarization causes calcium to move across the T-tubule into the sarcoplasmic reticulum and trigger the release of more activator calcium from the sarcoplasmic reticulum. Ford and Podolsky (1972b) used direct measurements of intracellular calcium movements in skinned muscle fibres as opposed to the indirect monitoring of contractile response (Endo, Tanaka and Ogawa, 1970; and Ford and Podolsky, 1970) to show that the release of calcium by calcium was a regenerative process and that the releasing effect was antagonized by increasing the magnesium concentration from 1 to 6mM. They calculated the normal magnesium concentration in the myofilament space of their fibres to be 1mM. Ford and Podolsky (1972a) considered their results to be consistent with a mechanism whereby

''during activation a small amount of calcium from the transverse tubules enters the myofilament space where it elicits the release of enough additional calcium from the (sarcoplasmic reticulum) to activate fully the myofilaments.'' Certainly as early as, 1959 Bianchi and Shanes detected calcium entry into skeletal muscle cells as a result of the action potential. If calcium enters, either to perform a function as the link, or just because it is there, it must be extruded again or as before the ''sinks'' will overflow.

The existence of mechanisms at the plasma membrane for extruding calcium from skeletal muscle cells has been indicated by Sulakhe, Drummond and Ng (1973), by Dipolo (1973b) and by Russel and Blaustein (1974). However, the isolated plasma membranes of Sulakhe et al. were prepared by lithium extraction, which has been clearly shown to have deleterious effects upon the calcium transporting ability of membranes (Repke and Katz, 1969). The results of Dipolo and of Russel and Blaustein have already been criticised in section B above. Thus although it has been clearly demonstrated that an active extrusion of calcium from the skeletal muscle cell is necessary in order to maintain normal function (Gilbert and Fenn, 1957; Bianchi and Shanes, 1959; Shanes and Bianchi, 1960; and Weiss and Bianchi, 1965) a calcium extrusion mechanism capable of maintaining both the free and bound calcium concentrations

in the muscle fibre has not been unequivocally demonstrated to be present in the plasma membrane.

D. Calcium regulation in myometrium.

Carsten (1969) isolated a fraction from cow uterus which she termed ''sarcoplasmic reticulum.'' She showed that this fraction was able to bind calcium in the presence of ATP and concluded that uptake and release of calcium by this fraction would be sufficient to regulate relaxation and contraction of the uterus. Recently Carsten (1973a, 1973b) has reported similar findings for pregnant cow uterus and pregnant human uterus.

Batra and Daniel (1972a) showed that both microsomal and mitochondrial fractions from rat myometrium could take up calcium in the presence of ATP. Their microsomal fraction was able to accumulate calcium from a 0.6uM solution · and they calculated that this calcium accumulation would suffice to account for relaxation of the myometrium.

Batra and Timby (1971) and Batra (1972) showed calcium uptake in both mitochondria and microsomal fractions from human myometrium and in, 1973, Batra reported that only the mitochondria from this tissue were capable of lowering the cytoplasmic calcium concentration

sufficiently to account for relaxation.

Clearly there are differences amongst reports as to what mechanism, microsomal or mitochondrial, is responsible for promoting relaxation in myometrium; although in some cases the tissue source is different and could account for the differences. In no case, however, is the contribution to microsomal calcium uptake from plasma membrane, smooth endoplasmic reticulum or rough endoplasmic reticulum known.

Arguments that there ought to be a calcium regulatory mechanism at the plasma membrane of the myometrium follow on logically from those used for skeletal muscle in Section C, and such a mechanism was proposed by VanBreemen, Daniel and VanBreemen (1966), who showed that the membrane was permeable to calcium but that the internal calcium concentration was far below the predicted value if its distribution were a passive process. Batra and Daniel (1970a and b) showed that a variety of metabolic poisons, when applied to the isolated rat uterus, lead to an increase in calcium content, an observation which is consistent with the hypothesis that cells of the rat uterus need to expend energy to maintain the intracellular calcium concentration at its resting level.

At least some calcium ion probably enters the myometrial cell in company with the action potential (Daniel, 1963; Marshall, 1963; Abe, 1970 and, 1971; Kuriyama, 1970; Anderson, Ramon and Snyder, 1971; Hodgson and Daniel 1973; and Szurszewski and Bulbring, 1973). If it does and even if there are calcium ''sinks'' inside the myometrial cell there should be some mechanism to extrude this extra calcium.

E. Tissue fractionation and membrane isolation.

The study of calcium regulation by subcellular bodies such as the mitochondria and sarcoplasmic reticulum involves the disruption of the cell and its segregation into separate components which can then be studied independently.

Critical evaluations of cell disruption and fractionation have recently been made by DePierre and Karnovsky (1973) and Wallach and Lin (1973). Although in both cases the authors were concerned specifically with the plasma membrane, several points that they made are salient to the whole process of isolation and characterization of subcellular fractions from muscle.

The first major problem in tissue fractionation which was pointed out by both DePierre and Karnovsky (1973) and

Wallach and Lin (1973) is that of the heterogeneity of the starting samples from which fractions are to be isolated. Heterogeneity in the starting sample may lead to erroneous conclusions about the properties of a given subcellular fraction from a given cell type if it is not eliminated or at least taken into consideration.

Wallach and Lin (1973) point out that although the pericellular pH is usually below 7.4 and although the pK values for many protein histidines lie near pH6.8 many workers have used buffers at pH7.4 for their fractionation procedures. The fact that small changes in pH around 7 can alter the physicochemical properties of certain membrane fragments (Wallach and Kamat, 1964; Wallach, Kamat and Gail, 1966) suggests that close attention should be paid to the pH at which fractionation is carried out. DePierre and Karnovsky (1973) however, cite several examples from which they claim that a higher pH of homogenizing medium leads to better fractionation and less aggregation of organelles.

Wallach and Lin (1973) point out that the ionic strength and divalent ion concentrations in fractionation media are often disregarded. Wallach and Lin (1973) hint that fractionation in the presence of divalent metal ions may afford greater protection to plasma membranes. DePierre and Karnovsky (1973) cite examples of sucessful

fractionation from the presence of EDTA in the homogenizing medium.

Of the techniques available for disruption of tissues the one which has been used almost exclusively in the study of calcium regulation in muscle is that referred to by Wallach and Lin (1973) as ''traditional shearing techniques.'' DeDuve (1971) has pointed out that very gentle homogenization of rat liver removes the outer membrane from 10% of the mitochondria which can be collected. However, the Waring blendor, which is extremely popular for muscle disruption (Ogawa et al., 1971), cannot be claimed to be gentle, and thus must be suspected of causing severe damage to several organelles.

The question of the methods for dividing the tissue into fractions once it has been homogenized has been discussed both by DePierre and Karnovsky (1973) and by Wallach and Lin (1973). Both sets of reviewers agree that sucrose density gradient separation has been quite successfully applied to tissue fractionation although it has been pointed out (DePierre and Karnovsky, 1973) that this approach establishes an osmolarity as well as a density gradient and such changes in osmolarity as are induced by this technique may be damaging to certain organelles. Both reviews are in favour of continuous as opposed to discontinuous sucrose density gradients since a

discontinuous gradient gives the illusion of a clear cut separation but is in fact subject to the production of artefacts due to the complex relation between the interfacial tensions between the steps in the gradient and the gravitational field.

The characterization of membrane fractions obtained by any fractionation technique is probably the most contentious area of the entire fractionation and characterization process.

Morphology is of little value as an index of characterization except for intact mitochondria, rough endoplasmic reticulum and nuclei which are quite distinctive; plasma membranes, smooth endoplasmic reticulum, sarcoplasmic reticulum and Golgi apparatus all form smooth vesicles upon disruption of the cell and so tend to look very much alike (DePierre and Karnovsky, 1973; and Wallach and Lin, 1973).

The most favoured of membrane markers are the intrinsic enzymes (Wallach and Lin, 1973). An intrinsic enzyme can be used as a marker if it can be shown to be localized at a specific site in the cell. Histochemical techniques have been used to indicate the presence of several such enzymes. However, there are some flaws in the histochemical techniques used which leaves the matter

somewhat open to doubt (DePeirre and Karnovsky, 1973; and Wallach and Lin, 1973). Firstly, fixation for microscopy or the agent used to identify the product of the enzymatic reaction may inhibit the enzyme under study differentially, such that a false impression of the localization at one site is generated; secondly, the agent used to identify the product of the enzymatic reaction may break down the substrate per se and falsely indicate that an enzyme is localized at more than one site; and thirdly, the substrate or the agent used to capture the enzymatic product might not diffuse to all the enzymatic sites which could again result in a false impression of a unique localization of the enzyme.

There is also the possibility that enzymes become bound during isolation to fragments with which they are not normally associated or that the enzymes are somehow activated or inactivated during the fractionation process, and the chance that the enzymes are concentrated in a certain functional component of the membrane and not spread homogeneously throughout the entire membrane.

Nevertheless as DePierre and Karnovsky (1973) point out, for solid tissues in which the plasma membrane must be disrupted before it can be studied there is little opportunity to use such techniques as membrane antigens, lectin receptors, virus receptors or hormone receptors as

primary agents of characterization since a change in orientation of some or all of the plasma membrane upon disruption would result in the production of anomalous results. The use of chemical labels to indicate the distribution of the plasma membrane seems usually to be handicapped by one or two diffuculties (Wallach and Lin, 1973); either the reagent will penetrate into other parts of the cell than the plasma membrane, if not on standing at least as a result ot redistribution on tissue disruption, or the agent will inhibit some aspect of the membrane function. Thus the enzyme marker technique is still quite extensively used, with reasonable success in many cases (DePierre and Karnovsky, 1973).

5'-Nucleotidase (EC 3.1.35) has often been used as a marker for plasma membrane in muscle systems (Hurwitz et al. , 1973; Kidwai, Radcliffe and Daniel, 1971; Kidwai et al. , 1973; and Meyer, 1973). There is abundant evidence from many types of tissue that 5'-nucleotidase is a very reliable marker for the plasma membrane (see DeDuve, 1971; and DePierre and Karnovsky, 1973). However, its localization in the plasma membrane has been challenged (Song, Kappas and Bodansky, 1969). Thus any attempt to characterize membrane fractions which uses 5'-nucleotidase as an indicator of the plasma membrane should be substantiated with other markers for the plasma membrane; the sodium+ potassium-activated magnesium-dependent ATPase

(EC 3.6.1.3), the potassium-stimulated p-nitrophenyl phosphatase and phosphodiesterase-I (EC 3.1.4.1) constitute reasonable substantiators (DePierre and Karnovsky, 1973).

The enzyme cytochrome-c oxidase (EC 1.9.1.3) became well accepted as a specific mitochondrial marker (Wallach and Lin, 1973) though it is restricted to the inner mitochondrial membrane. Azide-sensitive ATP-dependent calcium uptake is also a marker for mitochondria (Fanburg and Gergely, 1965).

Although several enzymatic markers have been used to designate material of endoplasmic reticular origin, none of them has proved really satisfactory (Wallach and Lin, 1973).

Coleman and Finean (1966), Benedetti and Emmelot (1968) and Thines-Sempoun et al. (1969) have presented evidence which indicates that the cholesterol/phosphlipid ratio of isolated plasma membranes is much higher than that of other membranes. However, Wallach and Lin (1973) find ''no evidence that the lipid composition of the plasma membranes is sufficiently characteristic to use it as a marker criterion. This appears particularly true for cholesterol which exchanges very easily with other lipoproteins and/or membranes.''

Thus although there are some clear guidelines in tissue fractionation and characterization, what is a ''correct'' or ''reliable'' approach to use is in many cases quite ambiguous. Several techniques which are used are based on incorrect assumptions and may be selected according to the personal prejudices of the particular investigator.

F. Aims of the research.

The aims of the research were to test the hypothesis that the plasma membrane, smooth endoplasmic reticulum, rough endoplasmic reticulum and mitochondria of uterine smooth muscle can all bind calcium in a manner compatible with a role in the process of relaxation, and that the calcium-accumulating mechanisms of these systems have differing properties from each other.

The hypothesis was to be tested by the isolation and identification of subcellular fractions of rat myometrium and by a study of the calcium uptake abilities of these fractions. Differences in the calcium uptake properties among the fractions would be investigated by studying the dependence on the free calcium concentration, the substrate dependence, the effect of barium, strontium and lanthanum ions which have been used in the study of

myometrial contractility (Daniel, 1963; Hodgson, Kidwai and Daniel, 1972; Hodgson and Daniel, 1973; and Marshall and Kroeger, 1973) and the effect of certain drugs which inhibit calcium uptake by the skeletal muscle sarcoplasmic reticulum (Balzer, Makinose and Hasselbach, 1968; and Balzer 1972).

Since it was not known how the isolation and fractionation procedures to be used would affect the calcium transporting abilities of the various membranes, subcellular fractions would be isolated from the much more widely studied skeletal muscle using the same techniques. Thus artefacts produced by the techniques might be discovered by comparing the data obtained for skeletal muscle with that already in the literature.

Chapter 2

<u>METHODS.</u>

The techniques used for the isolation and study of
subcellular fractions of rat myometrium were essentially
similar to those used for rat skeletal muscle. In this
chapter the methods used for myometrium will be given in
detail in section A and will not be repeated for skeletal
muscle; differences, however, will be pointed out in
section B which is devoted to skeletal muscle.

A. <u>Rat myometrium.</u>

1) Preparation of membrane fractions.

All centrifugation steps to be described were carried
out in Beckman L2-65B or L3-40 ultracentrifuges between 0
and 4°C.

a) Obtaining the cell-free homogenate.

15 to 30 female Wistar rats, weighing 160 to 200g
each, were killed by a blow on the head. The rats were
pre-treated for 2 days previously to the experiment with
500ug of diethylstilboestrol (dissolved in peanut oil) by

subcutaneous injection. Diethylstilboestrol treatment was performed so that all the uteri would be in the same state of hormonal control, i. e., under oestrogen domination, and to produce hyperplasia and hypertrophy and so increase the possible yield of material per uterus. The two uterine horns were rapidly removed and placed in ice-cold 8% sucrose, 40mM histidine solution at pH7.0 (pH was adjusted by addition of hydrochloric acid and this solution shall be referred to hereafter as sucrose-histidine solution). Individual uterine horns were trimmed of fat, and slit open with scissors, the endometrium and most of the circular muscle were separated from the longitudinal muscle using a scalpel and a pair of forceps; this entire process was carried out on a filter paper moistened with sucrose-histidine solution and maintained at approximately 4°C on the stage of a thermoelectric cold plate (Thermoelectrics Unlimited,Inc.). The longitudinal muscle was placed in ice-cold sucrose-histidine solution and the endometrium and circular muscle were discarded. Removal of endometrium and circular muscle resulted in a more homogenous starting material although there would still undoubtedly be fibroblasts mixed in with the smooth muscle cells.

When all uteri had been stripped in this way the accumulated longitudinal muscles were drained, blotted with filter paper until just moist and divided into lots

each of which was comprised of the longitudinal muscle from 10-12 uteri. The lots were placed in homogenization tubes made of cellulose nitrate, were finely minced with scissors and the volume in the tube was made up to 30ml with ice-cold sucrose-histidine solution. The contents of each tube were homogenized twice for 10 seconds at 15,000rpm in a Polytron PT20 (Kinematic GmbH) homogenizer which was fitted with an electronic time switch. During the entire procedure the tubes were kept on ice. An homogenizing medium of pH7.0 was chosen since this value seemed to be in the area which would produce the least amount of shock to both intracellular and extracellular material upon tissue disruption, it also happens to be in the range which Wallach and Lin (1973) think is most appropriate. A non-ionic, non-chelating medium was used since it was hoped to use fractions prepared this way to estimate the amount of bound divalent cations in each fraction with as little influence as possible from external circumstances (Janis and Daniel, unpublished).

The homogenate was then transferred to centrifuge tubes, with washings of ice-cold sucrose-histidine solution and centrifuged at 1,000xg (Average) for 15 minutes in a Beckman 60Ti rotor. This procedure removed fibrous material, whole nuclei, unhomogenized tissue and contractile proteins; the supernatant was named the cell-free homogenate. The cell-free homogenate was the

starting material from which both the mitochondrial fraction and the density gradient fractions were prepared. The steps involved in these two preparations are shown in Figure 1 and are outlined in b and c below. Initially only density gradient procedures (those starting to the left in Figure 1) were used, but normally both the mitochondrial fraction and the density gradient fractions were prepared from the same starting material; rarely only mitochondrial fractions were prepared (procedures starting to the right in Figure 1).

b) Obtaining the mitochondrial fraction.

The cell-free homogenate obtained by the procedures described above was centrifuged at 10,000xg (Av) for 10 minutes in a Beckman 60Ti rotor, the supernatant was used to obtain the membrane fractions by density gradient separation (see following). The pellets, which were each composed of material derived from 10-12 uteri, and the number of which varied with the number of animals killed, were resuspended in 10ml each of sucrose-histidine solution and rehomogenized by giving 8-10 gentle strokes in a loose-fitting teflon-glass homogenizer. The resuspended material was centrifuged at 1,000xg (Av) for 10 minutes in a Beckman 65 rotor and the supernatant from this spin was centrifuged at 10,000xg (Av) for 10 minutes in the same rotor. The pellet was resuspended as before

CELL-FREE HOMOGENATE

10,000xg/10 min

SUPERNATANT   SEDIMENT

114,000xg/30 min   1,000xg/10 min

SUPERNATANT   SEDIMENT   SUPERNATANT   SEDIMENT
IS SOLUBLE   LAYERED ON   DISCARDED
FRACTION   GRADIENT

                                      10,000xg/10 min

                              SUPERNATANT   SEDIMENT
                              DISCARDED

117,000xg/120 min   10,000xg/10 min

                              SUPERNATANT   SEDIMENT IS
                              DISCARDED   MITOCHONDRIAL
                                                 FRACTION

FRACTION
FROM
GRADIENT

10,000xg/10 min

SUPERNATANT   SEDIMENT IS
                                               "b" FRACTION

113,000xg/30 min

SUPERNATANT   SEDIMENT IS
DISCARDED   "a" FRACTION

Figure 1. Flow diagram for the isolation of subcellular fractions from rat myometrium.

and centrifuged at 10,000xg (Av) for 10 minutes in the 65 rotor. The pellet was the mitochondrial fraction and was resuspended and rehomogenized in 2 to 10ml of ice-cold sucrose-histidine solution depending on the final protein concentration required.

c) Obtaining the fractions by density gradient separation.

The supernatant from the 10,000xg spin of the cell-free homogenate was centrifuged at 114,000xg (Av) for 30 minutes in a Beckman 60Ti rotor and the sediment of the material originally derived from 10-12 uteri was suspended in 3ml of sucrose-histidine solution and rehomogenized in a loose-fitting teflon-glass homogenizer giving 8-10 gentle strokes. This homogenate was then layered on top of the sucrose density gradient, which had been equilibrated at 4ºC, by means of a Pasteur pipette. The density gradient was comprised of 4ml of 45% sucrose on top of which were carefully layered 3ml of 33% sucrose followed by 3ml of 28% sucrose. The density gradient preparation was then centrifuged for 2 hours at 111,700xg (Av) in a Beckman SW40 (swinging bucket) rotor. At the end of this spin the fractions were removed from the gradient by Pasteur pipette and transferred to fixed-angle centrifuge tubes where the sucrose concentration was slowly diluted, with continuous gentle shaking, to 8% with deionized water and the volume was adjusted to 10ml by

adding sucrose-histidine solution. These tubes were centrifuged at 10,000xg (Av) for 10 minutes using the Beckman 65 rotor, the pellets from these spins were designated as ''b'' fractions. The supernatants from the spins yielding the ''b'' fractions were centrifuged at 113,000xg (Av) in the Beckman 65 rotor for 30 minutes and these pellets were termed the ''a'' fractions. For the number and location of fractions taken from the gradient see Chapter 3. All pellets obtained were resuspended in 1-5ml of sucrose-histidine solution depending on the final protein concentration required, and were homogenized by giving 5 gentle strokes in a loose-fitting teflon-glass homogenizer. The fractions were kept on ice until used. Protein concentration was determined by the method of Lowry, Rosebrough, Farr and Randall (1951) using bovine serum albumin dissolved in sucrose-histidine solution as the standard.

2) Characterization of the fractions.

In order to determine the subcellular origin of the various fractions obtained, the technique of assaying for certain marker enzymes which has been commonly used in similar types of work on various muscles (Hurwitz et al. , 1973; Katz et al. , 1970; Kidwai, Radcliffe and Daniel, 1971; Kidwai, Radcliffe, Duchon and Daniel, 1971; Kidwai et al. , 1973; McNamara, Sulakhe and Dhalla, 1971; Meyer,

1973; Severson, Drummond and Sulakhe, 1972; and Sulakhe et al. , 1971) was employed.

a) Plasma membrane markers.

5'-Nucleotidase was used as a plasma membrane marker in these studies and the technique of Song and Bodansky (1967), which involves the measurement of inorganic phosphate released from 5'-AMP, was applied to measure its activities. Where necessary (see Chapters 3 and 4) 5'-nucleotidase activity was corrected for non-specific phosphatase activity which was measured using beta-glycerolphosphate as substrate (Dixon and Purdom, 1954). Such corrections have been routinely made (see Dixon and Purdom, 1954; and Song and Bodansky, 1967) on the assumption that the non-specific phosphatases break down beta-glycerolphosphate and 5'-AMP at the same rate. The assumption is based upon the demonstration by Dixon and Purdom (1954) that a wide variety of phosphate compounds were broken down at the same rate by non-specific phosphatase enzymes. However, there is no absolute guarantee that the rates of breakdown of beta-glycerolphosphate and of 5'-AMP by the non-specific phosphatases in the systems which I examined were exactly the same. The corrections thus make the values obtained somewhat approximate in absolute value, but more accurate than total values obtained without consideration for large

non-specific phosphatase activities.

Because of the criticism cited in Chapter 1 of the applicability of 5'-nucleotidase as a plasma membrane marker, this enzyme alone was not relied upon. Thus the potassium-activated, ouabain-sensitive para-nitrophenyl phosphatase was measured by the method of Kidwai, Radcliffe and Daniel (1971) except that pH7.4 was used and 1mM ouabain was used to determine ouabain sensitivity. As a final marker for plasma membrane phosphodiesterase-I was measured by the technique of Touster et al. (1970).

In all cases above, the inorganic phosphate liberated was measured by the method of Fiske and SubbaRow (1926) using 2,4-diaminophenol hydrochloride as the reducing agent (Skou, 1957).

b) Mitochondrial markers.

The enzyme cytochrome-c oxidase was relied upon to indicate the presence of mitochondrial material. Since cytochrome-c oxidase activity is located in the inner mitochondrial membrane, monitoring of this activity would not give an indication of contamination by the outer mitochondrial membrane. Cytochrome-c oxidase activity was measured by the method of Cooperstein and Lazarow (1951).

A further indicator of mitochondrial material is the azide-sensitive ATP-dependent calcium uptake (Fanburg and Gergely, 1965) and measurement of this property was used to supplement the observations of cytochrome-c oxidase activity. Measurement of calcium uptake will be described in detail below; 0.5mM sodium azide was used to determine azide sensitivity.

c) Endoplasmic reticulum markers.

In the absence of a truly acceptable marker for the endoplasmic reticulum, a fraction of membraneous composition with little or no plasma membrane and mitochondrial enzyme activities would be classed as endoplasmic reticulum.

As a further aid to characterization, measurement of the cholesterol:phospholipid ratio, which has been carried out in other muscle systems (Kidwai, Radcliffe and Daniel, 1971; Kidwai et al. , 1973; and Severson, Drummond and Sulakhe, 1972) was considered. However, the lack of any definitive knowledge as to what this ratio should be for each membrane in the myometrial cell, plus the apparent fact that both phospholipid and cholesterol do occur in all the membranes in which I had primary interest in separating, would provide an insoluble problem for the characterization of, what were likely to be, not

rigourously pure fractions.

The difficulty of solving the last mentioned problem coupled with the criticisms of the use of lipids as markers made by Wallach and Lin (1973) and cited in Chapter 1, strongly argues against the usefulness of the measurement of the cholesterol:phospholipid ratio as a primary index of characterization.

d) Electron microscopy.

Electron microscopy was used in this case to determine the vesicular nature of the fractions, to check that fractions showing mitochondrial marker enzyme activities were composed of intact mitochondria and, as will be seen in Chapter 3, it also proved useful in distinguishing between rough and smooth endoplasmic reticulum.

For electron microscopy the fractions were not suspended in sucrose-histidine solution after the final centrifugation step, but were immediately fixed in buffered glutaraldehyde. The fractions were later post fixed in osmium tetroxide. Sections were stained with uranyl acetate and lead citrate and viewed and photographed on a JEM 7A electron microscope.

3) Measurement of calcium uptake.

Calcium uptake was measured using an adaptation of the filtration technique (Martonosi and Feretos, 1964). The details of the method used in this study are as follows:

a) Reaction mixtures.

Reaction mixtures normally had a final volume of 1ml; however, for studies of the time course of calcium uptake, when several aliquots required to be sampled at different times, the reaction mixture volumes were 5 or 6ml. The composition of the reaction mixture was: 100mM potassium chloride; 5mM magnesium chloride; 0.1mM calcium chloride, labelled with 0.4 microCurie per ml [45]calcium chloride; 40mM imidazole and 10-50ug of protein depending on the fraction being assayed. The pH of the reaction mixture was always 7.0 as adjusted by addition of hydrochloric acid monitored on a Beckman Expandomatic pH meter. Imidazole at 40mM was chosen as the buffer since Murphy and Koss (1968) showed clearly that under these conditions and with the amounts of phosphate expected to be released in these experiments it would buffer the pH adequately and so prevent changes in calcium concentration from being caused by hydrogen ion induced release of calcium from EGTA complexes (see later). For ATP-dependent uptake studies 5mM disodium ATP was present in the reaction

mixture, for free calcium controlled experiments the calculated concentrations of potassium EGTA to give the required free calcium concentrations (see later) were added. 5mM potassium oxalate, 0.5mM sodium azide or ions as chloride salts, various drugs and substrates other than ATP were added as indicated.

b) Incubations.

Uptake reactions were normally carried out at 37⁰C with reaction tubes placed in a shaking water bath. Reactions were started by adding protein (0.1ml for every 1ml of final reaction mixture) after a pre-incubation time of 5 minutes. Immediately upon addition the contents of the reaction tubes were mixed on a Vortex-Genie mixer and returned to the water bath. The amounts of protein added were adjusted from fraction to fraction depending upon preliminary estimates of their ATPase activities, so that changes in ATP concentration would not prove limiting to calcium uptake. Thus for the major fractions (see Chapter 3 for full description and terminology) these were 15-20ug protein per final ml of reaction mixture for PM, 30-50 per final ml for SER and RER and 50-100 per final ml for MITO. However in ATP-free conditions 100-120ug per final ml were used for all fractions.

c) Filtration.

Uptake reactions were stopped (normally after 10 minutes but in time course experiments as indicated) by first of all thoroughly mixing the contents of the reaction tubes on a Vortex-Genie mixer and then withdrawing 0.8ml with a Pipetman (Gilson) automatic pipette. The 0.8ml aliquot was filtered through 25mm diameter cellulose nitrate filter disks with a pore size of 0.45 microns (Matheson-Higgins Co., Inc.) which were held on a Millipore 3025 filtration manifold and kept under vacuum using a Sargent Welch vacuum pump. Filtration took 2-3 seconds and was followed by a wash with 10ml of 8% sucrose (which took 5-10 seconds), 40mM imidazole solution at pH7, to remove unbound calcium. This wash was necessary to give consistent results (see Sulakhe, Drummond and Ng, 1973) and washing with the non-ionic medium produced as consistent results as did washing with 100mM potassium chloride or the complete reaction mixture minus ATP and ^{45}calcium (see also Weiner and Cha, 1972).

Prior to filtration of the aliquot all filters were washed with 10ml of 100mM potassium chloride solution followed by 10ml of the sucrose-imidazole solution previously described. This procedure resulted in low background counts (i.e., low counts on the filter paper

when reaction mixtures to which no protein but an equivalent volume of sucrose-histidine solution was added, were filtered) which were not obtained in its absence (see also Palmer and Posey, 1970). Presumably potassium ion bound to negative sites on the filter that might otherwise have attracted calcium ion; the sucrose-imidazole served merely to wash off excess potassium ion. Even using this treatment there was still considerable variation in the blanks. The main sources of variation came from the free calcium concentration of the medium being filtered and the batches of filters used. Some batches of filters bound up to twice as much calcium as did others, hence filters from the same batch were always used within an experiment. Under these conditions blanks did not vary by more than 10%. When solutions of 85uM free calcium were used blanks normally fell within the range of 800-1,200cpm whilst under experimental conditions, i.e., with added protein present, counts were greater than 3,000 per minute.

d) Scintillation counting.

Filters through which appropriate blanks containing no added protein but the same volume of suspending medium had been filtered, together with the experimental filters, were removed and placed in scintillation vials to which 10ml of scintillation fluid (Bray, 1960) were added. Also 0.1ml aliquots of reaction mixture from blank tubes before

filtration were placed in vials and scintillation fluid was added. The vials were counted to 2% accuracy in a Beckman LS 330 or Picker Nuclear Liquimat liquid scintillation counter. Quenching as measured by the external standards ratio method was negligible and no correction ever needed to be made.

e) Calculation of calcium uptake.

All computations of calcium uptake were made by the use of a PDP8/E digital computer (Digital Equipment Corporation) controlled by both Fortran and Focal programmes. Normally punched tape output from the scintillation counter was processed in Fortran to make its format acceptable to Focal; the Focal programme was actually used to compute the calcium uptake. From counts in the aliquots of reaction mixture and given the amounts of calcium in the reaction mixture the programme calculated the specific activity of the calcium in the medium. The blanks were averaged to give an estimate of calcium bound to the filter paper and this was subtracted from the counts in experimental conditions, which were taken to represent calcium bound to the filter paper + calcium bound to the protein which was trapped on the filter paper, hence one aquired the counts associated with the protein. From the specific activity of the calcium, the counts associated with protein and the amount of

protein per sample, the uptake of calcium in micromoles per g protein was calculated. In the earliest experiments two and in later experiments four blanks were run for each experimental condition.

4) Control of the free calcium concentration.

The free calcium concentration in the medium at pH7.0 is dependent on the concentrations of ATP, calcium ion, magnesium ion and EGTA in the reaction mixture (Katz, Repke, Upshaw and Polascik, 1970). Normally the amounts of ATP, calcium and magnesium in the reaction mixtures were the same and a simple Focal computer programme was used to indicate how much EGTA was required to give a specified calcium concentration given all the other conditions. The programme was based upon the equations given by Katz et al. . The constants used to describe the various complexes were as follows: the log dissociation constant for the fourth hydrogen ion of ATP, -6.97 (O'Sullivan and Perrin, 1964); the binding constant for magnesium and ATP, 6×10^4 (Godt, 1974); the binding constant for CaATP, 2.5×10^4 (Godt, 1974); and the binding constant for CaEGTA, 1.35×10^6 (Godt, 1974). When no EGTA was added to the reaction mixture in the presence of ATP the free calcium concentration was calculated to be 17uM. The amounts of EGTA to be added in the absence of ATP to produce various free calcium concentrations could also be

calculated, as could the amounts of calcium to be added in the absence of EGTA but in the presence of ATP.

The correctness of the absolute values of the free calcium concentrations to be given throughout this thesis is entirely dependent upon the correctness of the constants which have been employed to compute these values. The most important constant as regards the value of the free calcium concentration produced is probably the binding constant for CaEGTA. The value for this constant used here, 1.35×10^6, is appropriate since the conditions under which it was calculated were extremely similar to those which I used.

5) Estimation of calcium-ATPase.

The composition of the reaction mixtures for the measurement of calcium-ATPase were similar to those for ATP-dependent calcium uptake. Reaction mixtures contained no radioisotope and sufficient EGTA to give a free calcium concentration of 1uM when calcium was added.

Basal ATPase activities were measured with no calcium added to the reaction mixture (i.e., in the presence of EGTA) and basal+calcium ATPase activities were measured in the same medium but with calcium added.

Incubations were as described for calcium uptake and were stopped by the addition of 1ml of cold 10% TCA and accompanied by thorough mixing. Inorganic phosphate liberated was measured as previously described.

6) Pre-treatment with lanthanum.

Some of the fractions were pre-treated with lanthanum in order to find the effect of this agent on calcium uptake.

The final pellets from the isolation of the fractions concerned were divided into two groups and suspended in sucrose-histidine (control) or sucrose-histidine containing the required concentration of lanthanum chloride (experimental), at 37ºC for 15 minutes. The suspended material was then recentrifuged at the normal force and time used to obtain the final pellet during the isolation procedure. The pellet obtained from this spin was washed twice with sucrose-histidine solution to remove unbound lanthanum. In each wash sucrose-histidine solution was applied from a Pasteur pipette and allowed to run down the tube and over the pellet after which it was carefully poured off. The pellet was then suspended in sucrose-histidine solution and treated as a normally obtained membrane fraction.

7) Age of fractions.

The measurements of cytochrome-c oxidase, calcium uptake and calcium-ATPase activities were always performed on freshly prepared fractions as soon after isolation as possible. Other enzyme determinations were carried out on fractions which had been frozen and stored at -20°C overnight.

B. Rat skeletal muscle.

Unless specifically noted in this section, methods used for skeletal muscle study were the same as described for myometrium.

1) Preparation of membrane fractions.

The back leg muscles from 1 or 2 female Wistar rats weighing 160-200g which had been killed by a blow on the head were quickly removed and placed in ice-cold sucrose-histidine solution. The muscles were trimmed of fat, nerve and connective tissue on moistened filter paper kept at 4°C on the stage of the thermoelectric cold plate. They were divided into lots of about 5g which were placed in cellulose nitrate homogenization tubes and finely minced with scissors. The mince was made up to 30ml with ice-cold sucrose-histidine solution and homogenized once

using the Polytron PT20 homogenizer at 15,000 rpm for 15 seconds. The homogenate with washings was transferred to centrifuge tubes and centrifuged for 10 mins at 600xg (Av) in a Beckman 21 rotor. The supernatant from this spin was the cell free homogenate. It was treated exactly as was the cell free homogenate of myometrium in order to obtain the various fractions. As far as quantities are concerned the material derived from 5g of original muscle was treated the same as the material from 10-12 uteri.

2) Characterization of the fractions.

In addition to 5'-nucleotidase, the sodium+ potassium-activated, ouabain-sensitive ATPase which has been used extensively in skeletal muscle fractionation procedures (Kidwai et al. , 1973; and Sulakhe et al. , 1971 and, 1973a) was measured in this study. The method used by Kidwai et al. (1973) was followed.

3) Measurement of calcium uptake.

This procedure was the same as for myometrium except that 30 second incubation times were normally used for the PM and SR fractions whilst 10 minute incubations were used for the MITO fractions (for designation of fractions see Chapter 4). 20 to 30ug of protein per ml of reaction mixture were used for the PM and SR fractions whilst 50 to

100ug per ml were used for the MITO fraction.

C. Materials used.

All water was distilled and then passed through a series of Barnstead ion exchange demineralizer columns so that its specific resistance on emerging was greater than 16 megohms per square centimeter. On standing the specific resistance decreased due to the absorbance of carbon dioxide from the atmosphere. The inorganic compounds used in calcium uptake and calcium-ATPase reaction mixtures were analytical grade, otherwise they were reagent grade. Most of the organic compounds used, except the drugs noted below, were obtained from the Sigma Chemical Corporation and all were of the highest purity obtainable. [45]Calcium chloride was obtained in lots of 1 milliCurie at regular intervals from the Amersham-Searle Corporation. The drugs which were used and their sources are as follows: chlorpromazine, Poulenc; diethyl stilbestrol, Sigma; D600 hydrochloride and verapamil (isoptin hydrochloride), Knoll; propranolol hydrochloride, Ayerst. Preparative solutions were normally made up in batches to serve for an entire week and were kept frozen when not in use. The density of sucrose solutions was checked using a Bausch and Lomb Refractometer. The various reaction mixtures were made up on the day of the experiment; however, stock solutions were made up every 1-

3 weeks and kept frozen when not in use. The radioisotope was diluted to 40 microCuries per ml with distilled water on arrival and was kept at 4°C.

Calcium uptake reaction mixtures had an osmolarity of 250 to 270mOsm as measured on a freezing point depression osmometer.

D. Statistical analysis.

Where necessary the significances of the differences between the results obtained under control and experimental circumstances were tested using Scheffe's test (Scheffe, 1959) and by taking p=0.05 as the cut off point for statistical significance. Significance or lack of significance of the results, measured in such a way, has been noted in the text or in the tables of Chapters 3 and 4.

Chapter 3

RESULTS OF EXPERIMENTS ON RAT MYOMETRIUM.

A. Results of density gradient centrifugation.

After being sedimented at 113,000xg for 30 minutes, then resuspended and centrifuged at 111,700xg for 120 minutes on the sucrose gradient, the homogenate became divided into six fractions. The numbering of these fractions and their locations on the gradient are shown in Table 1. The further differential centrifugation of these fractions (excepting the pellet) resulted in their subdivision into ''a'' and ''b'' fractions. ''a'' fractions sedimented at 113,000xg when centrifuged for 30 minutes, but not at 10,000xg for 10 minutes. ''b'' fractions sedimented at 10,000xg when centrifuged for 10 minutes.

The protein yields amongst the fractions obtained, together with the non-particulate soluble fraction, are shown in Table 2 expressed as a percentage of the total protein. As an average of 6 experiments fraction 1a yielded 0.37 mg protein per g wet weight of longitudinal muscle. Fractions 1b, 2b, 3b and, 4b were small and since preliminary experiments showed no difference between 1b

TABLE 1

Location of the various subcellular fractions
from rat myometrium on the sucrose-density
gradient.

Fraction   location on gradient

   1   at interface of loading medium and 28% band

   2   in 28% band

   3   at interface of 28% and 33% band

   4   in 33% band

   5   at interface of 33% and 45% band

   6   as a pellet

TABLE 2

Distribution of protein from cell-free homogenate after purification procedure on rat myometrium.

Fraction	% total protein recovered
1a	2.48±0.46
2a	1.36±0.29
1b+2b	0.68±0.13
3a	0.76±0.09
4a	0.64±0.12
3b+4b	0.45±0.11
5a	0.53±0.11
5b	2.2±0.31
6	4.8±1.20
Soluble fraction	86.10±2.01

Number of preparations=4.

and 2b or between 3b and 4b, 1b and 2b were usually combined as were 3b and 4b.

B. Characterization of the subcellular fractions obtained.

The subcellular fractions obtained were subjected to analysis of electron microscopic appearance, content of enzyme activities and preliminary calcium uptake properties in order to provide a basis for characterization of their subcellular origin.

1) Electron microscopic appearance.

Fractions 1a, 2a, 3a and 4a all had very similar appearances when studied by electron microscopy. There were a large number of vesicular structures, being more uniform in size in the lighter fractions, and there was also, in all cases, a small quantity of non-vesicular material. Plates 1 and 2 show electron micrographs of fractions 1a and 4a. Plate 3 shows fraction 5a; it too was vesicular but as can be seen almost all the vesicles were bounded by electron-dense granules. The electron-dense granules were taken to be ribosomes and these electron micrographs strongly suggested that fraction 5a was composed substantially of particles originating from the rough endoplasmic reticulum. Fractions 1b, 2b, 3b and

Plate 1. Electron micrograph of fraction 1a from rat myometrium. Magnification is x 20,000.

Plate 2. Electron micrograph of fraction 4a from rat
myometrium. Magnification is x 20,000.

<u>Plate 3.</u> Electron micrograph of fraction 5a from rat myometrium. Magnification is x 20,000.

4b, like their respective ''a'' fractions again showed similar appearances to one another. Plate 4 shows an electron micrograph of fraction 2b. There were some normal vesicular structures but the majority of these fractions was composed of flattened tadpole-like structures. Fraction 5b, as shown in Plate 5, was composed of irregularly shaped, irregularly sized vesicles as well as an amount of non-vesicular material. The pellet contained very occasional unbroken mitochondria, collagen and other non-vesicular material.

2) 5'-Nucleotidase activity.

Preliminary experiments conducted using both 5'-AMP and beta-glycerolphosphate as substrate showed that there was only a very small amount of non-specific phosphatase present in all the fractions. Hence it was considered that for this system a study of the distribution of ability to break down 5'-AMP amongst the subcellular fractions would be an accurate measure of the distrubution of 5'-nucleotidase activity. The results of measurements of 5'-nucleotidase activities in the these fractions are shown in Table 3. The highest specific activities of 5'-nucleotidase appear in fractions 1 and 2 and usually the lighter the fraction, the higher the activity. The activity in fraction 3a is less than half of that in fractions 1 and 2 and from 3a onwards there is a gradual

Plate 4. Electron micrograph of fraction 2b from rat myometrium. Magnification is x 20,000.

<u>Plate</u> <u>5.</u> Electron micrograph of fraction 5b from rat
myometrium. Magnification is x 20,000.

TABLE 3

5'-Nucleotidase activities of subcellular fractions from rat myometrium.

Fraction	Specific activity
Homogenate	7.8±0.77 (10)
1a	110.5±3.4 (32)
2a	91.5±4.2 (30)
1b+2b	142.6±5.8 (13)
3a	49.2±3.8 (29)
4a	28.0±1.5 (30)
3b+4b	35.0±3.1 (14)
5a	14.4±1.1 (21)
5b	10.3±0.7 (26)

Activity expressed as micromoles Pi released/mg protein/hour. Numbers in brackets represent number of preparations.

further decline in activity with increasing density of
fraction.

3) Potassium ion-activated, ouabain-sensitive
phosphatase.

The amount of ouabain-sensitive, potassium-stimulated
phosphatase that was found in each of the subcellular
fractions is shown in Table 4. As with 5'-nucleotidase
this enzyme is concentrated in fractions 1 and 2 and less
than half of the activity of fractions 1 and 2 appears in
fraction 3, whereafter there is a considerable decrease in
activity with increase in fraction density. Fractions
1b+2b and 3b+4b were not assayed for this enzyme.

4) Phosphodiesterase-I activity.

The distribution of activity of the phosphodiesterase-
I enzyme amongst the subcellular fractions is shown in
Table 5. Again after the isolation procedure the specific
activity of this enzyme is highest in fractions 1 and 2,
being nearly twice as high in 1 as in 2. Activity falls
off with increasing density of fraction.

TABLE 4

Potassium-stimulated, ouabain-sensitive phosphatase activities in subcellular fractions of rat myometrium.

Fraction	Specific activity
Homogenate	0.07±0.13 (4)
1a	3.16±0.25 (6)
2a	2.15±0.19 (3)
3a	1.05±0.15 (3)
4a	0.25±0.28 (5)
5a	0.20±0.11 (5)
5b	0.03±0.13 (3)

Values expressed as micromoles Pi released/mg protein/hour. Numbers in brackets represent number of preparations.

TABLE 5

Phosphodiesterase-I activities in subcellular fractions of rat myometrium.

Fraction	Specific activity	
Homogenate	1.20±0.09	(7)
1a	16.70±0.54	(13)
2a	8.50±0.64	(5)
1b+2b	19.00±1.7	(4)
3a	4.75±0.65	(2)
4a	3.93±0.59	(12)
3b+4b	4.53±0.92	(3)
5a	2.53±0.14	(12)
5b	1.38±0.16	(4)

Values expressed as micromoles Pi released/mg protein/hour. Numbers in brackets represent number of preparations.

5) Cytchrome-c oxidase activity.

Fraction 5b proved to be extremely rich in the mitochondrial marker enzyme cytochrome-c oxidase. 5b was so rich in this enzyme in contrast to the other subcellular fractions studied that the specific activities of the other fractions have been expressed as a percentage of the 5b activity and are shown in Table 6. An almost reverse distrubtion to that of the plasma membrane markers is seen for this enzyme. Its highest activity is in fraction 5b, there is just over one quarter of the 5b activity in fraction 5a and a gradual reduction in activity from then onwards with decreasing density.

6) Calcium uptake activity.

Calcium uptake activity of the subcellular fractions was studied to determine which if any of them were capable of accumulating calcium in the presence of ATP, in excess of that which became bound when ATP was absent. Calcium uptake was studied in the presence of oxalate so as to give an indication of whether calcium transport into the vesicles was occurring, and in the presence of azide in order to determine the involvement of mitochondrial ATPases in the uptake mechanism. Table 7 gives a summary of the results from this complex series of experiments.

TABLE 6

Cytochrome-c   oxidase   activities   in
subcellular   fractions   of   rat
myometrium.

Fraction	Specific activity
1a	1.0±0.2 (12)
2a	3.7±0.5 (12)
1b+2b	7.7±0.6 (8)
3a	7.5±1.3 (12)
4a	13.5±1.4 (12)
3b+4b	17.5±3.5 (6)
5a	29.9±3.6 (9)
5b	100 (12)

Activities expressed as a % of the
cytochrome-c oxidase specific activity
in fraction 5b. Numbers in brackets
represent number of preparations.

TABLE 7

Calcium uptake by various subcellular fractions of rat myometrium.

Fraction	No addition	ATP	ATP+ potassium oxalate	ATP+ sodium azide
		Additions to reaction mixture		
Homogenate	0.9±0.5	6.1±1.1	11.2±6.0	1.8±0.1*
1a	5.2±1.0	22.0±2.7	29.2±3.6*	21.9±3.0
2a	6.1±0.9	20.1±1.9	29.4±3.1*	20.6±1.4
1b+2b	5.6±0.9	28.0±8.4	30.7±5.1	14.0±4.5*
3a	4.2±0.6	17.5±1.9	26.8±4.2*	14.4±2.1
4a	5.0±0.6	17.1±1.8	35.8±4.8*	11.9±0.8*
3b+4b	3.7±0.2	37.9±5.3	45.6±3.7	29.9±1.6*
5a	4.9±1.0	16.9±1.2	48.3±8.9*	10.5±2.5*
5b	2.8±0.2	36.7±18.2	33.8±15.0	4.3±1.1*

Values expressed as mean ± s.e. of micromoles calcium accumulated per g protein during an incubation time of 10 minutes. For no addition number of preparations=6 in all cases. In cases where additions were made number of preparations=6 for 1b+2b and 3b+4b, 10 for 3a and 5a, 12 for 1a and 4a, and 14 for 2a. * Denotes values significantly different from those obtained in the presence of ATP alone.

A striking observation is that all fractions showed an ATP-dependent calcium uptake property. The ATP-dependent calcium uptake was enhanced by oxalate in all the ''a'' fractions. Enhancement by oxalate was greatest, being 3 times and 2 times increased respectively, in 5a and 4a and was in general increased with increasing density of ''a'' fraction. Azide had very little effect on the lighter fractions but increasing inhibition appeared with increasing density of fraction. ATP-dependent calcium uptake in fraction 5a was inhibited by almost 50% and in fraction 5b by more than 95% in the presence of azide. These results show that the distribution of azide-sensitive calcium uptake resembles that of cytochrome-c oxidase activity.

C. Designation of the subcellular fractions obtained.

From the concentration of their activities in the first two fractions it was clear that 5'-nucleotidase, potassium-stimulated, ouabain-sensitive phosphatase and phosphodiesterase-I activities indicated a very substantial enrichment of these two fractions by material derived from the plasma membrane. The cytochrome-c oxidase levels in fractions 1 and 2 were very low which indicated little contamination with material of inner mitochondrial origin, and the neglible effect of azide on calcium uptake by these fractions indicated little

contamination by fragments of mitochondria able to take up calcium. In the absence of a satisfactory method for estimating the amount of endoplasmic reticulum present, it is not possible to say how much these fractions were contaminated by material from this source.

If, in the absence of a reliable marker enzyme for endoplasmic reticulum, one were to assume that 5'-nucleotidase, potassium-stimulated, ouabain-sensitive phosphatase and phosphodiesterase-I were exclusive to the plasma membrane, then the fractions which best represented endoplasmic reticulum would be those with the lowest activities of the above three enzymes, whilst also being low in mitochondrial enzymes. The fractions with the lowest content of plasma membrane marker enzymes were fractions 4 and 5. From cytochrome-c oxidase studies fraction 5b was quite clearly mainly of mitochondrial derivation. Electron micrographic study had already indicated that much of fraction 5a originated from the rough endoplasmic reticulum, although it was shown by cytochrome-c oxidase activity and by the effect of azide on calcium uptake to be somewhat contaminated by mitochondrial protein.

Fraction 4a thus became the best representative of material derived from the smooth endoplasmic reticulum; it was low in activity of both plasma membrane and

mitochondrial marker enzymes, was vesicular and few of the vesicles were bounded by ribosomes. However, to what extent this fraction might have been composed of rough endoplasmic reticulum which had lost its ribosomes cannot be estimated. Clearly fraction 3 was a mixture of material derived from the plasma membrane and the endoplasmic reticulum.

Thus the fractions chosen to best represent plasma membrane, smooth endoplasmic reticulum and rough endoplasmic reticulum were fractions 1a, 4a and 5a respectively and shall be referred to hereinafter as PM, SER, and RER. Clearly no fraction represented intact mitochondria.

The combined fractions 1b+2b and 3b+4b were interesting both morphologically and enzymatically; especially fraction 1b+2b which had the highest specific activity of 5'-nucleotidase and of phosphodiesterase-I of all the fractions isolated but was, however, more contaminated with material of mitochondrial origin than was either fraction 1a or 2a. The low yields of these fractions and sheer economy of the study obviated further investigation of their calcium uptake properties.

D. <u>Examination</u> <u>of</u> <u>the</u> <u>properties</u> <u>of</u> <u>the</u> <u>mitochondrial</u>
<u>fraction</u> <u>obtained</u> <u>by</u> <u>differential</u> <u>centrifugation.</u>

It was clear from the studies reported in section C
above that the sucrose density gradient procedure resulted
in the separation of mitochondrial marker enzyme activity
from the morphological appearance of intact mitochondria.
Since this study sought to examine the calcium uptake
properties of smooth muscle mitochondria, it was thought
necessary to prepare them in such a way that they would
remain as nearly as possible intact. For this reason a
mitochondrial fraction was prepared by the differential
centrifugation technique described in Chapter 2.

Enzyme activities of this fraction are shown in Table
8. As can be seen plasma membrane marker enzymes had low
activities but not as low as those in fraction 5b from the
density gradient preparation. Similarly, cytochrome-c
oxidase activity in this fraction was considerably lower
than in fraction 5b of the density gradient. However,
electron micrographs of the mitochondrial fraction (see
Plate 6) showed it to be composed to a large extent of
intact mitochondria. It is possible that fraction 5b from
the sucrose-density gradient was purified inner
mitochondrial membrane

Calcium uptake experiments on the mitochondrial

TABLE 8

Enzyme activities of the mitochondrial
fraction from rat myometrium.

5'-nucleotidase   18.0 ±1.5[1]   (19)

Potassium-stimulated,
ouabain-sensitive
phosphatase   0.3 ±0.1[1]   (4)

Phosphodiesterase-I   1.51±0.13[1]   (3)

Cytochrome-c
oxidase   31.4 ±3.7[2] (5)

[1]Activities expressed as micromoles Pi
released/mg protein/hour.

[2] Activity expressed as a % of the
cytochrome-c oxidase specific activity
of fraction 5b. Numbers in brackets
represent number of preparations.

fraction confirm its mainly mitochondrial origin. Results are shown in Table 9. The uptake in the presence of ATP by this fraction was much higher than that by any of the other fractions and, despite the fact that it showed a small oxalate effect, the ATP-dependent calcium uptake was almost completely inhibited (more than 95%) by azide. For studies on calcium uptake, this fraction, isolated by conventionally accepted methods for the preparation of mitochondria, could be considered to be of mitochondrial origin. This fraction will be referred to as MITO.

E. Calcium uptake by the major fractions.

Once the subcellular fractions had been obtained and had been designated as being derived from certain subcellular loci as described in the preceeding sections, the ''major fractions'' (i.e., those most closely resembling plasma membrane, smooth endoplasmic reticulum, rough endoplasmic reticulum and mitochondria) were studied for their ability to accumulate calcium under certain conditions which might serve to elucidate their respective roles in relaxation of the smooth muscle.

1) Time course of calcium uptake.

In the preliminary calcium uptake experiments described in section B, part 6 above and section D above,

TABLE 9

Calcium uptake by the mitochondrial
fraction from rat myometrium.

Additions to reaction mixture

No addition   2.7± 0.3

ATP   116.4±24

ATP +
potassium oxalate   144.9±30*

ATP +
sodium azide   7.4± 1.3*

Values expressed as mean ± s.e. of
micromoles calcium accumulated per g
protein during an incubation time of
10 minutes. Number of preparations=8.
* Denotes values significantly
different from that obtained in the
presence of ATP alone.

all the major fractions had been shown able to accumulate calcium from a 17uM solution when 5mM ATP was present in the incubating medium. The first task in more clearly defining the calcium uptake properties of these fractions was to determine the time course of calcium uptake for the major fractions. The results of these determinations are shown in Figure 2.

The upper curve in Figure 2 shows that there is considerable similarity amongst what will referred to as the vesicular fractions (PM, SER, and RER). They all show a very rapid phase of calcium uptake up to 30 seconds which is similar in magnitude for all fractions. The rate of net calcium uptake is greatly reduced after the first 30 seconds; however, net calcium accumulation continues in all fractions until a steady-state value is reached at around 15 minutes. The steady-state values of calcium accumulation attained by the vesicular fractions fall within the range of 19 (SER) to 31 (RER) micromoles calcium per g protein.

In contrast to the vesicular fractions, the lower curve in Figure 2 shows that the steady-state net calcium uptake by mitochondria under these conditions is 4 to 5 times as great as that for the vesicular fractions.

Figure 2. <u>Time</u> <u>course</u> <u>of</u> <u>calcium</u> <u>accumulation</u> <u>by</u> <u>the</u> <u>major</u> <u>fractions</u> <u>of</u> <u>rat</u> <u>myometrium.</u> The incubation medium contained 5mM ATP and a free calcium concentration of 17uM. Upper curve, filled triangles represent RER, filled circles PM, open circles SER; standard errors not shown to avoid clutter. Lower curve MITO. The length of the vertical bars projecting above and below the points represent the standard error of the mean. All results are from 6 preparations.

2) Effect of free calcium concentration on
calcium uptake.

Since the part played by a subcellular structure in
the process of relaxation is limited by its ability to
accumulate calcium from solutions of low free calcium
concentration (see Chapter 1), the ability of the major
fractions in this regard was studied. An incubation time
of 10 minutes was chosen as giving a good balance between
steady-state of uptake, not too much ATP breakdown and a
measureable amount of calcium taken up at low calcium
concentrations. The free calcium concentrations were
buffered to the required level using EGTA, as described in
Chapter 2, making adjustments for the presence and absence
of ATP.

Figures 3 and 4 show the results of these
experiments. Again the vesicular fractions show
remarkable similarities as illustrated by Figure 3A, B, C.
All showed an increase in uptake upon the addition of ATP
at 0.03uM free calcium and this difference between ATP-
dependent and ATP-independent calcium uptake an increased
with increase in free calcium concentration. The uptake
mechanism did not appear to be saturated even at 33uM free
calcium.

By contrast, Figure 3D shows that the mitochondria

Figure 3. Effect of calcium concentration on calcium uptake by the major fractions from rat myometrium. Incubations were for 10 minutes. A is PM, B SER, C RER and D MITO. Open circles in the presence and filled circles in the absence of ATP. The length of the vertical bars projecting above and below the points represent the standard error of the mean. Results are from 7 to 18 preparations.

Figure 4. <u>Effect of calcium concentration on calcium
uptake by MITO fraction from rat myometrium.</u> Incubations
were for 10 minutes. Smaller scale to show full extent of
ATP-dependent calcium uptake by this fraction. The length
of the vertical bars projecting above and below the points
represent the standard error of the mean. Results are
from 12 to 18 preparations.

were poor at accumulating calcium from 0.03 and 0.3uM free calcium solutions, in these cases ATP-dependent and ATP-independent calcium uptake could usually not be resolved. However, a massive and drastic jump in calcium-accumulating ability of mitochondria takes place over the range of 0.3 to 1uM free calcium. At 1uM, mitochondria are far superior to the vesicular fractions in the amount of calcium they can accumulate. This drastic increase in ability to accumulate calcium is continued past 1uM and is such that it cannot be represented on the same scale as that of the vesicular fractions. The full calcium-dependence curve for ATP-dependent uptake by mitochondria is shown in Figure 4; like the vesicular fractions, saturation does not appear to be reached even at 33uM free calcium.

3) Effect of products of ATP breakdown on calcium uptake

Irrespective of the mechanism(s) of ATP-dependent calcium uptake in the subcellular fractions being studied preliminary experiments showed that the fractions were all capable of breaking down ATP to ADP, AMP and inorganic phosphate under the conditions to be used for calcium uptake studies. Thus it was of interest and importance to find out what influence ADP and AMP might have on the calcium-accumulating ability of the major fractions.

Table 10 shows the effect of the presence of both ADP and AMP at two concentrations on the amount of calcium accumulated by the major fractions in ten minutes from a 17uM free calcium solution. AMP had little or no effect upon calcium uptake in any case. The 1mM concentration of ADP produced a slight, though not significant, decrease in calcium uptake in PM and SER fractions only, whilst the 5mM concentration inhibited uptake to a varying extent in all fractions. The results indicate a further reason to keep ATP breakdown to a minimum when attempting to measure either maximal rates or maximal amounts of calcium uptake.

4) Effect of the ionic environment on calcium uptake from a solution of 0.3uM free calcium concentration.

Since in the intact muscle, the plasma membrane is bounded on both sides by media of different ionic compositions, it was of interest to investigate the possible effect of these different environments upon calcium uptake from low calcium solutions; that is, under the kind of conditions which might be operative for relaxation. Similarly it was also thought necessary to find out if the plasma membrane would behave differently from the endoplasmic reticulum in this regard, and to what extent the physiologically important univalent cations, sodium and potassium, influenced the uptake process.

TABLE 10

Effect of products of ATP-hydrolysis on calcium uptake by subcellular fractions of rat myometrium.

	ADP		AMP	
	1mM	5mM	1mM	5mM
PM	89±5	80±4*	92±7	103±9
SER	90±4	59±4*	97±2	94±2
RER	102±3	63±8*	106±2	103±5
MITO	115±5	79±16	107±20	100±7

Values expressed as mean ± s.e. of % of calcium uptake in the presence of added product. Incubation time 10 minutes. Number of preparations=4. * Denotes values significantly different from 100%.

PM, SER and RER fractions were incubated for 10 minutes in media in which all sodium and potassium ions of the normal reaction mixture had been iso-osmotically replaced with sucrose and which contained ATP and a free calcium concentration of 0.3uM buffered with EGTA. Uptakes at 10 minutes were compared with those from solutions containing 150mM potassium chloride and 10mM sodium chloride, to represent the intracellular solution, 5mM potassium chloride and 110mM sodium chloride to represent the extracellular solution, and with the normal reaction mixture. The results are illustrated in Table 11.

As can be seen there is a generalized depression of calcium uptake activity with increase in ionic strength and in all cases the medium with the high sodium content caused a greater depression of calcium uptake than did the high potassium containing medium.

There were no differences in the direction of the effect of the different ionic environments on calcium uptake amongst the fractions studied; that is uptake was not increased by high sodium in the PM fractions whilst being depressed in the SER and RER.

However, the decreases in uptake shown by PM and SER in the solution most closely resembling the internal

TABLE 11

Effect of the ionic environment on calcium uptake by subcellular fractions of rat myometrium.

	150mM KCl 10mM NaCl	5mM KCl 110mM NaCl	100mM KCl 10mM NaCl
PM	60±10*	49± 4*	85± 9
SER	55±12*	53± 7*	102± 4
RER	87±17	55±12*	94±23

Values expressed as mean ± s.e. of % of calcium uptake in control solution, which contained 200mM sucrose.
Incubation time 10 minutes. Number of preparations=4. * Denotes values significantly different from 100%.

cellular environment indicate that certain corrections would have to be made to estimates of calcium uptake from low calcium solutions obtained from the normal reaction mixture used throughout this work when trying to apply them to estimate the role of plasma membrane and smooth endoplasmic reticulum in muscle relaxation.

5) Initial rate of calcium uptake.

To be certain of its role in relaxation even in the more slowly contracting and relaxing smooth muscles, one must know to what extent a subcellular structure can accumulate calcium over a very brief period of time (perhaps 1 or 2 seconds). However, by the technique used in this study, measurements of calcium uptake over a part of a second were not possible. In order to get a clearer estimate of what the initial rate of calcium uptake might be, the reaction was slowed down by operating at 20°C. Even if the rate of uptake by the fractions was not linear between 0 and 20 seconds at 20°C, samples taken at 20 seconds would at least provide an approximate underestimate of the true initial rate of calcium uptake. The free calcium-concentration used for this experiment was 1uM. The results obtained are shown in Table 12. PM, SER and RER took up almost identical amounts of calcium under these conditions whilst MITO took up only about half as much as the other fractions.

TABLE 12

Calcium   uptake   by   subcellular
fractions of rat myometrium at 20°C.

PM	1.3±0.2
SER	1.2±0.2
RER	1.5±0.2
MITO	0.7±0.2

Values expressed as mean ± s.e.
(Number   of   preparations=27)   of
micromoles calcium taken up per g
protein in 20 seconds.

6) Effect of various substrates on calcium uptake.

Since calcium can be accumulated by skeletal muscle sarcoplasmic reticulum under the influence of a number of substrates (see Chapter 1) one can ask whether ATP is the only compound that will act as substrate for calcium accumulation by the myometrial fractions, or are the other nucleotides as effective? Must a substrate be a nucleotide or can any substance with an energy-rich phosphate-ester bond be used as substrate? ITP and GTP were chosen as nucleotides and acetylphosphate (AcP) and para-nitrophenyl phosphate (PNPP) as other phosphate esters. Uptakes in the presence of these agents were compared to uptakes in the presence of ATP using a 10 minute incubation and a 17uM free calcium concentration. The results are shown in Table 13. In no case, except of course for that in the presence of ATP, was uptake significantly greater than that in the absence of added substrate (measured in a separate series of experiments), indicating that under the conditions used PM, SER, RER and MITO all required ATP in order to take up extra calcium. Exactly the same type of results was obtained when oxalate was present in the reaction mixture, as is shown in Table 14.

Since mitochondria from various sources take up calcium by the use of succinate as a respiratory

TABLE 13

Calcium uptake by subcellular fractions of rat
myometrium in the presence of various substrates
at a concentration of 5mM.

	GTP	ITP	AcP	PNPP	None
PM	21 ± 5	26 ±6	27 ± 8	25 ±19	35±5
SER	28 ± 7	32 ±7	26 ±11	21 ± 4	36±3
RER	16 ±10	30 ±5	25 ±11	11 ± 3	30±3
MITO	2 ± 0	3 ±0	2 ± 0	1 ± 0	3±1

Values expressed as mean ± s.e. of % of calcium
uptake in the presence of 5mM ATP. Number of
preparations=3.

TABLE 14

Calcium uptake by subcellular fractions of rat myometrium in the presence of various substrates at a concentration of 5mM and of potassium oxalate at a concentration of 5mM.

	GTP	ITP	AcP	PNPP	None
PM	14± 8	26±5	36±13	13±7	17±5
SER	20± 7	19±7	26±11	21±4	19±8
RER	16±10	20±5	25±11	11±3	14±6

Values expressed as mean ± s.e. of % of calcium uptake in the presence of 5mM ATP and 5mM oxalate. Number of preparations=3.

substrate, the effect of succinate on calcium accumulation by the MITO fraction from rat myometrium was studied in this regard. It was particularly necessary to see if succinate could increase the uptake by mitochondria at low (i.e., 0.3uM) free calcium concentration, and thus make mitochondria more important for the calcium sequestration of relaxation. The results are shown in Table 15. Clearly under these conditions succinate did not act as a substrate for calcium uptake by the MITO fraction per se at any free calcium concentration. However, succinate together with ATP produced a higher uptake than did ATP alone at free calcium concentrations of 1 and 17uM, but at the calcium concentration of 0.3uM, even the combined substrates were no better than ATP alone. In experiments further to the ones indicated in Table 15, no increase in calcium uptake by the MITO fraction was seen when 1mM of organic phosphate was added to a reaction mixture containing succinate as the sole substrate; these results are shown in Table 16.

7) Effect of various drugs on calcium uptake.

The effect of certain drugs on calcium uptake by the major fractions is shown in Table 17. The free calcium concentration used in the experiments was 17uM and, as can be seen, all the fractions were inhibited to some degree by these drugs. In every case MITO showed the greatest

TABLE 15

Effect of succinate on calcium uptake by mitochondria from rat myometrium.

	ATP 5mM	Succinate 5mM	ATP 5mM+ Succinate 5mM
17uM calcium	143.8±71.7	2.2±0.5	249.0±14.2
1uM calcium	14.8± 1.8	0.7±0.4	55.4±14.2
0.3uM calcium	1.1± 0.8	0.7±0.5	0.6± 0.8

Values expressed as mean ± s.e. of micromoles calcium accumulated per g protein during an incubation time of 10 minutes. Number of preparations=3. In separate experiments, uptake in the absence of substrate was found to be 3.8±1.2 at 17, 0.8±0.2 at 1, and 0.9±0.2 at 0.3uM free calcium, units as above and number of preparations in this case was 8.

TABLE 16

Effect of 1mM organic phosphate on calcium
uptake by mitochondria from rat myometrium in
the presence of 5mM succinate.

	No Pi added	1mM Pi added
17uM calcium	5.1±1.1	5.5±1.3
1uM calcium	1.6±0.5	1.0±0.3
0.3uM calcium	0.9±0.8	0.6±0.2

Values expressed as mean ± s.e. of micromoles
calcium accumulated per g protein during an
incubation time of 10 minutes. Number of
preparations=3.

TABLE 17

Effect of some drugs on calcium uptake by subcellular fractions of rat myometrium.

	Chlorpromazine 0.1mM	D600 1mM	Propranolol 1mM	Verapamil 0.1mM	1mM
PM	47±7	58±7	56±5	100±15[1]	44±10
SER	31±6	32±7	32±8	92±9[1]	32±11
MITO	2±0	11±4	20±4	69±19[1]	5±2

Values expressed as mean ± s.e. of % of calcium uptake in the absence of added drug. Incubation time 10 minutes. Number of preparations=4. All values are significantly different from 100% except [1].

sensitivity to the drugs and SER was more sensitive than PM (RER was not investigated). The results of similar experiments using concentrations of 10 and 0.1uM chlorpromazine and verapamil and incubation times of 5 minutes (pre-steady-state) are shown in Table 18.

8) Effect of divalent cations on ^{45}calcium uptake.

The effects of adding calcium, barium and strontium (as their chlorides) at a concentration of 1mM on ^{45}calcium uptake are shown in Table 19. Before the addition of the divalent ion the free calcium concentration was 2.5uM buffered only with ATP, to avoid complex interactions between the divalent ion and EGTA; interactions between divalent ions and ATP could not be avoided. In all systems added calcium was, predictably, extremely effective in reducing the uptake of labelled calcium from the medium. Strontium too, was quite effective in this regard, though in PM, SER and RER it was not as effective as calcium itself. However, in MITO strontium was at least as effective as calcium in reducing the uptake of labelled calcium. Barium was the least effective of the three ions tested.

TABLE 18

Effect of some drugs, at low concentration on calcium uptake by subcellular fractions of rat myometrium.

	Chlorpromazine		Verapamil	
	10uM	0.1uM	10uM	0.1uM
PM	97±12	99±13	101± 7	102± 8
SER	102±11	87± 4	100±14	101±19
RER	81± 2*	110±10	98± 3	85± 7
MITO	66± 2*	113±20	84±10	111± 5

Values expressed as mean ± s.e. of % of calcium uptake in the absence of added drug. Incubation time 10 minutes. Number of preparations=3. * Denotes values significantly different from 100%.

TABLE 19

Effect of addition of divalent cation on ^{45}calcium uptake by subcellular fractions of rat myometrium.

	Calcium chloride 1mM	Strontium chloride 1mM	Barium chloride 1mM
PM	1±1	10±3	53±4
SER	5±1	16±2	72±10[1]
RER	5±2	13±2	70±6
MITO	5±1	3±1	51±7

Values expressed as mean ± s.e. of % of ^{45}calcium uptake when no extra divalent cation added, i.e. with free calcium concentration of 2.5uM. Incubation time 10 minutes. Number of preparations=3. All values except [1] significantly different from 100%.

9) Effect of lanthanum on ^{45}calcium uptake.

Because of complex solubility reactions taking place between lanthanum, liberated inorganic phosphate and ATP, it was not possible to study the effects of lanthanum ion on calcium uptake by the method which was described in section 8. However, the effect of lanthanum on calcium uptake was thought important enough to merit attention and it was tackled in a different way. The technique for pre-treating the fractions with lanthanum has been described in Chapter 2. In this series of experiments SER and RER were combined into one fraction which has been called ER.

Concentrations of 0.1, 1 and 10mM lanthanum chloride were used to pre-treat the fractions. Unfortunately in all cases approximately 50% of the protein was lost as a result of the lanthanum pre-treatment. The protein loss was found not to be an artefact produced by the presence of lanthanum in the protein assay. The reasons for this lanthanum chloride-induced loss of protein are not known. The vesicles tended to aggregate after lanthanum treatment and uptake in the control fractions of the ER was reduced as a result of the rehomogenization procedure. Perhaps because of the protein loss, perhaps for other reasons, the results obtained in these experiments were extremely variable and really defy analysis. Uptake of ^{45}calcium from a 2.5uM solution was measured at 10 minutes and

lanthanum treated fractions were compared with control. The results are shown in Table 20. There was a significant decrease in calcium uptake by the ER fraction at 1mM but not at 10mM lanthanum, whereas there was a significant decrease in calcium uptake by the PM fraction at 10mM but not at 1mM.

F. Calcium-ATPase in the major fractions.

To determine whether a calcium-ATPase that might be responsible for the ATP-dependent calcium uptake measured was present in these fractions, the fractions were incubated with ATP both in the presence and in the absence of added calcium. EGTA was present in both instances to remove any contaminating calcium in the first case and to produce a free calcium concentration of 1uM in the second. The amount of inorganic phosphate released in each case is shown in Table 21. All fractions apparently released more inorganic phosphate, i.e., split more ATP when calcium was present, than they did in its absence. The order for the amount of ''extra-splitting'' was PM greatest, then RER, then SER with MITO being least. Significantly more extra-splitting, however, occurred only in the PM and RER fractions.

TABLE 20

Effect of pre-incubation with lanthanum on ^{45}calcium uptake by subcellular fractions of rat myometrium.

	Lanthanum chloride 0.1mM	Lanthanum chloride 1mM	Lanthanum chloride 10mM
PM	144±88 (3)	202±65 (5)	39±15 (3)*
ER	111±15 (3)	49± 3 (3)*	77±19 (3)

Values expressed as a % of the calcium uptake in the control fractions which were not pre-incubated with lanthanum. Incubation time 10 minutes. * Denotes values significantly different from 100%. Numbers in brackets represent number of preparations.

TABLE 21

Effect   of   calcium   on   ATP   hydrolsis   by
subcellular fractions of rat myometrium.

	PM	SER	RER	MITO
Added calcium	116±4	46±3	34±3	23±5
No added calcium	104±5	41±3	26±1	21±4
Difference	12*	5	8*	2

Values expressed as micromoles Pi released /mg
protein/10   minutes.   *   Denotes   that   the
difference is significant.

Chapter 4

RESULTS OF EXPERIMENTS ON RAT
SKELETAL MUSCLE.

A. Results of density gradient centrifugation.

As a result of density gradient centrifugation the skeletal muscle homogenate was divided into 6 fractions. The numbering of these fractions and their locations on the gradient were the same as for myometrium, shown in Table 1. Each fraction was subdivided into ''a'' and ''b'' by differential centrifugation. ''b'' fractions sedimented at 10,000xg when centrifuged for 10 minutes; ''a'' fractions sedimented when the supernatant of the spin yielding the ''b'' fractions was centrifuged at 113,000xg for 30 minutes. The percent protein yields of the fractions obtained from rat skeletal muscle are shown in Table 22.

In the experiments on rat skeletal muscle, interest was focussed mainly on investigating the calcium uptake properties of the fractions which most resembled plasma membrane, and in comparing these with the properties of the fractions most resembling what has been commonly accepted as sarcoplasmic reticulum. It was useful to

TABLE 22

Distribution of protein from cell-free
homogenate   after   purification
procedure on rat skeletal muscle.

Fraction	% total protein present in cell-free homogenate.
1a	0.89±0.05 (5)
1b+2b	0.09±0.03 (2)
3a	0.81±0.16 (5)
3b+4b	0.16±0.03 (4)
5a	0.54±0.63 (2)
5b	2.79±0.98 (4)
Soluble Fraction	77.34±3.14 (5)

Numbers in brackets represent number
of preparations.

compare these two fractions with a mitochondrial fraction obtained by differential centrifugation. No attempt was made to find the calcium uptake properties of every fraction that was isolated and consequently some of the bands have been neglected.

In preliminary experiments fraction 1a appeared to resemble plasma membrane most closely whilst fraction 3a seemed to be derived from the sarcoplasmic reticulum, as there was little difference between it and fraction 4a. Fraction 5b was very rich in the mitochondrial marker cytochrome-c oxidase and appeared to be of mitochondrial origin.

After the preliminary experiments, a more detailed study as to the origin of the various fractions was carried out and it is detailed in the following section.

B.   Characterization of the subcellular fractions obtained.

The morphological appearances of the fractions were examined by electron microscopy. 5'-Nucleotidase and the sodium+ potassium-activated, ouabain-sensitive ATPase were used as enzyme markers for the plasma membrane whilst cytochrome-c oxidase was used as a marker for mitochondria and was supplemented by observations of azide-sensitive

calcium uptake.

1) Electron microscopic appearance.

Plates 7 and 8 show electron micrographs of skeletal muscle fractions 1a and 3a respectively. Both fractions are almost entirely vesicular in nature and there is very little difference in gross appearance between them.

2) 5'-Nucleotidase activity.

In preliminary experiments using both 5'-AMP and beta-glycerol phosphate as substrates, a considerable amount of non-specific phosphatase was detected to a varying degree in the fractions. Hence it was necessary to express 5'-nucleotidase activities as the difference between phosphate release when 5'-AMP was used as substrate and that when beta-glycerol phosphate was used as substrate. The rates of hydrolysis of beta-glycerol phosphate and of glucose-6-phosphate were the same in all fractions (Janis 1974). The results are shown in Table 23. There is an almost fourfold increase in specific activity of this enzyme in the 1a fractions as compared to fraction 3a; some 5'-nucleotidase was detectable in the soluble fraction.

Plate 7. Electron micrograph of PM fraction from rat skeletal muscle. Magnification is x 62,000.

Plate 8. Electron micrograph of SR fraction from rat skeletal muscle. Magnification is x 62,000.

TABLE 23

Activities   of   5'-nucleotidase   in
subcellular   fractions of rat skeletal
muscle.

Fraction	Activity
Homogenate	1.01±0.28 (4)
1a	4.10±1.03 (4)
3a	1.01±0.49 (3)
Soluble fraction	0.58±0.28 (3)

Values   expressed   as   micromoles   Pi
released/mg protein/hour. Numbers in
brackets   represent   number   of
preparations.

3) Sodium, potassium-activated, ouabain-sensitive ATPase.

All fractions exibited an increased splitting of ATP when sodium and potassium ions were added to an incubation medium which already contained magnesium. There was little difference amongst the fractions in this increase in ATP splitting indicating that a sodium+ potassium-activated, ouabain-sensitive ATPase was present in all the fractions. However, there were differences in the extent to which the sodium- and potassium-enhanced activities were susceptible to 1 mM ouabain, a dose of drug which did not significantly affect the splitting of ATP prior to the addition of sodium and potassium. Thus fraction 1a had a ouabain-sensitive, sodium+ potassium-activated ATPase which released 71.9±12(n=5) micromoles of inorganic phosphate per mg protein per hour, whilst that of fraction 3a liberated 22.7±11(n=5) micromoles per mg per hour, an increase in specific activity of more than 3 times from fraction 3a to fraction 1a. Because of high background ATPase activity ouabain sensitivity could not be detected in the homogenate.

4) Cytochrome-c oxidase activity.

As was mentioned earlier fraction 5b proved to be extremely rich in cytochrome-c oxidase activity. The

activities of the other fractions were measured and expressed as a percentage of the specific activity of fraction 5b; they are shown in Table 24. There is relatively low contamination by cytochrome-c oxidase in both fractions which were of particular interest, namely 1a and 3a. Fraction 4a had more than twice the specific activity of cytochrome-c oxidase of fraction 3a.

5) Preliminary calcium uptake studies.

Calcium uptake was studied both in the presence and in the absence of ATP; with oxalate and with azide, the results from fractions 1a and 3a together with those for the mitochondrial fraction prepared by differential centrifugation and from the cell-free homogenate are shown in Table 25."

Both fraction 1a and fraction 3a took up more calcium in the presence of ATP than in its absence; the increase in uptake produced by ATP was twice as much in 3a as it was in 1a. In the presence of oxalate both fractions 1a and 3a greatly increased the amount of calcium they took up, 1a taking up as much as 3a.

TABLE 24

Activities of cytochrome-c oxidase in subcellular fractions of rat skeletal muscle.

Fraction	Specific activity
Homogenate	2.7±0.2 (4)
1a	2.2±0.4 (5)
3a	7.0±0.3 (4)
4a	15.2±1.0 (5)
5b	100 (5)

Values expressed as a % of cytochrome-c oxidase specific activity in fraction 5b. Numbers in brackets represent number of preparations.

TABLE 25

Calcium uptake by various subcellular fractions
of rat skeletal muscle.

Fraction	Additions to reaction mixture.			
	No addition	ATP	ATP+potassium oxalate	ATP+ sodium azide
Homogenate	1.1±0.9	5.1±1.1	165.2±20*	5.7±0.5
1a	2.3±0.5	8.9±2.6	1137±248*	7.7±2.8
3a	2.3±0.3	15.9±5.5	1109±326*	15.5±3.0
Mitochondrial	3.3±1.6	180.9±29.6	203.5±50	3.7±0.5*

Values expressed as mean ± s.e. of micromoles
calcium taken up per g protein during an
incubation time of 10 minutes from a free
calcium concentration of 17uM. Number of
preparations=5. * Denotes values significantly
different from those obtained in the presence of
ATP alone.

C. Designation of the subcellular fractions obtained.

Fraction 1a was able to take up calcium from a solution of 17uM free calcium concentration in the presence of ATP. This ability to take up calcium was greatly increased when oxalate was present in the reaction mixture. The above properties have normally been attributed to sarcoplasmic reticulum of skeletal muscle. However, fraction 1a also contained the greatest concentration of plasma membrane marker enzymes that could be obtained using the proceedures which have been described. It also had a very low content of the mitochondrial marker enzyme. Clearly fraction 1a was enriched in material derived from the plasma membrane and is the fraction which most closely represents plasma membrane amongst those obtained; thus it will be referred to as PM. Fraction 3a was low in plasma membrane marker enzyme activity and had some, but not an excessive amount, of contamination by the mitochondrial marker enzyme. It also showed azide-insensitive calcium uptake properties that are normally attributed to sarcoplasmic reticulum. Fraction 3a will be referred to as SR.

D.   Examination of the properties of the mitochondrial
fraction obtained by differential centrifugation.

Electron micrographs of this mitochondrial fraction
showed it to be composed mainly of intact mitochondria
(see Plate 9). This fraction had no detectable ouabain-
sensitive,   sodium,potassium-activated   ATPase and had
41.3±3.0% of the cytochrome-c oxidase specific activity of
the 5b fraction from the density gradient preparation. In
calcium uptake studies it was able to accumulate large
amounts of the ion from a 17uM solution under the
influence of ATP; this uptake was, however, only slightly
increased by oxalate and was nearly totally abolished by
azide.

The mitochondrial fraction was thus indeed composed
mainly of mitochondrial material and will be referred to
as MITO.

E. Calcium uptake by the major fractions.

After being isolated and identified,   the   major
fractions, namely PM, SR and MITO, were studied in greater
detail as regards their ability to take up calcium, in
order to more clearly establish the roles of plasma
membrane, sarcoplasmic reticulum and mitochondria in
calcium regulation in skeletal muscle.

Plate 9. Electron micrograph of MITO fraction from rat skeletal muscle. Magnification is x 15,000.

1) Time course of calcium uptake.

The time courses of calcium uptake by the three major
fractions under a variety of circumstances are shown in
Figure 5. From a 17uM free calcium solution both PM and
SR took up calcium extremely rapidly, maximum net uptake
being attained in less than 60 seconds after which there
was a gradual fall with time. SR took up more calcium at
all times than did PM. The MITO fraction was very
different from PM and SR both in time course and amounts
of calcium taken up. The presence of oxalate had a marked
multiplying effect on calcium uptake by PM and SR. Two
things are particularly noteworthy under these
circumstances. Firstly, the reduction in net labelled
calcium associated with the PM and SR membranes after 60
seconds in its absence, did not occur in the presence of
oxalate. Secondly, whereas the SR was better at taking up
calcium than the PM in the absence of oxalate, the
presence of oxalate made the two fractions almost
identical in ability to accumulate the ion. The effect of
oxalate on the MITO fraction was very small and has been
left off the figure so as not to add confusion. Figure 5D
shows the time course of calcium uptake from a 0.3uM
solution. The general shape of the time course curve was
the same as from 17uM free calcium in the case of PM and
SR. SR was still able to accumulate a large amount of
calcium and there was a wider difference between it and PM

Figure 5. <u>Time courses of calcium accumulation by the major fractions of rat skeletal muscle.</u> Filled circles represent SR, open circles PM and filled triangles MITO. A, B and C are from 17uM free calcium concentrations whilst D is from 0.3uM. In B only, 5mM oxalate is present. The length of the vertical bars projecting above and below the points represent the standard error of the mean. All results are from 5 preparations.

at this concentration than at 17uM. In comparision to PM
and SR uptake by MITO was very small.

2) Effect of free calcium concentration on calcium
uptake.

The ability to take up calcium from solutions of
different free calcium concentrations both in the presence
and in the absence of ATP was studied using EGTA as a
buffer to control the free calcium concentration.
Incubation times of 30 seconds for PM and SR and 10
minutes for MITO were used. The results are shown in
Figure 6. Both PM and SR were able to accumulate calcium
in the presence of ATP from solutions of 0.03uM free
calcium, and the amount taken up generally increased with
increase in calcium concentration, although PM and SR both
appeared to be reaching a plateau as regards amount taken
up at the free concentration of 17uM. MITO was different,
as is shown by Figures 6C and 6D. MITO ATP-dependent
calcium uptake was almost non-existent at free
concentrations of 0.03 and 0.3uM. However, between 0.3
and 1uM a large jump in the ability to accumulate calcium
occured; from 1uM onwards an increase in calcium
concentrations produced increased calcium uptake.

Figure 6. <u>Effect of calcium concentration on calcium uptake by the major fractions of rat skeletal muscle.</u> A is PM, B SR, C MITO and D MITO on a smaller scale. Open circles are in the presence and closed circles in the absence of ATP. The length of the vertical bars projecting above and below the points represent the standard error of the mean. The results are from 4 preparations for PM and SR and 4 to 10 for MITO.

3) Effect of the ionic environment on calcium uptake from a solution of 1uM free calcium concentration.

The influence of the ionic composition of the reaction mixture was studied. Uptakes from a medium in which all sodium and potassium in the normal reaction mixture was iso-osmotically replaced by sucrose were compared to those in the normal uptake medium and to those in ones which contained high potassium and high sodium. A free calcium concentration of 0.3uM was used in order to determine how the above conditions might be effective at low calcium concentrations. The results are shown in Table 26. Although an increase in ionic strength produced a generalized depression of calcium uptake, the effects were only significant in the PM fraction.

4) Initial rate of calcium uptake.

All three major fractions were studied for the amount of calcium they could take up at 20°C in 20 seconds. The free calcium concentration used was 1uM and the results are shown in Table 27. Under these conditions the greatest amount of calcium was taken up by SR followed by PM followed by MITO, which took up just over one tenth as much as did PM.

TABLE 26

Effect of the ionic environment on calcium uptake by subcellular fractions of rat skeletal muscle.

	150mM KCl 10mM NaCl	5mM KCl 110mM NaCl	100mM KCl 10mM NaCl
PM	90±13	69± 9*	76±8*
SR	79±9	75±12	86±9

Values expressed as mean ± s.e. of % of calcium uptake in control solution which contained 200mM sucrose. Incubation time 30 seconds. Number of preparations=4. * Denotes values significantly different from 100%.

TABLE 27

Calcium   uptake   by   subcellular
fractions of rat skeletal muscle at
20°C.

PM	SR	MITO
15.6±1.7	23.7±2.5	1.6±0.1

Values expressed as mean   ±   s.e.
(Number   of   preparations=27)   of
micromoles calcium taken up per g
protein in 20 seconds.

5) Effect of various substrates on calcium uptake.

As shown in Table 28 the substrates GTP, ITP and acetlyphosphate were able to support calcium uptake in both PM and SR to the same extent that ATP did. Para-nitrophenyl phosphate supported calcium uptake in PM and SR but not as well as ATP. No differences were observed between PM and SR as regards substrate dependence for uptake. In MITO, however, none of these substrates were capable of supporting calcium uptake to any noteworthy degree. Table 29 shows what happened to calcium uptake in the presence of oxalate when the various substrates were used to replace ATP. All uptakes were greater than in the absence of ATP; however, none of the substrates could support calcium uptake under these conditions as well as ATP. There were no differences between PM and SR either as regards extent of uptake produced or order of potency of the substrates tested.

Table 30 shows that succinate failed to support calcium uptake by MITO at any of the calcium concentrations tested. The combination of ATP and succinate did not increase calcium uptake from solutions of 0.3 and 1uM free calcium and only slightly increased it from the 17uM solution.

TABLE 28

Calcium uptake by subcellular fractions of rat
skeletal muscle in the presence of various
substrates at a concentration of 5mM.

	GTP	ITP	AcP	PNPP
PM	125±6	100±5	101±4	47±3
SR	112±6	92±7	112±22	43±6
MITO	4±0	1±0	5±0	1±0

Values expressed as mean ± s.e. of % of calcium
uptake in the presence of 5 milliMolar ATP. PM
and SR incubated for 30 seconds, MITO for 10
minutes. Number of preparations=4.

TABLE 29

Calcium uptake by subcellular fractions of rat
skeletal   muscle   in   the   presence   of   various
substrates at a concentration   of   5mM,   and   of
potassium oxalate at 5mM.

	GTP	ITP	AcP	PNPP	None
PM	19±5	28±0	4±1	1±0	0.2±0.0
SR	24±1	28±1	3±1	1±0	0.2±0.0

Values   expressed as mean ± s.e. of % of calcium
uptake in   the   presence   of   5mM   ATP   and   5mM
oxalate.   Incubation   time 30 seconds. Number of
preparations=4.

TABLE 30

Effect of succinate on calcium uptake by
mitochondria from rat skeletal muscle.

	ATP 5mM	Succinate 5mM	ATP 5mM+ Succinate 5mM
17uM calcium	112.9±49.8	1.2±0.4	145.0±42.7
1uM calcium	33.6±17.9	0.2±0.1	38.4±4
0.3uM calcium	0.6±0.5	1.4±1.7	0.3±0.4

Values expressed as mean ± s.e. of micromoles
calcium accumulated per g protein during an
incubation time of 10 minutes. Number of
preparations=3. In separate experiments, binding
in the absence of substrate was 3.0±1.6 at 20,
2.3±0.6 at 3, and 0.7±0.9 at 0.3uM calcium,
units as above.

6) Effect of drugs on calcium uptake.

When the drugs chlorpromazine, verapamil and propranolol at concentrations of 0.1 mM were tested for their effects on calcium uptake by the major fractions from calcium solutions of 1uM, as shown in Table 31. Only chlorpromazine had any significant effect. Uptake in all fractions was reduced, and mitochondria were more susceptible than PM and SR.

The results obtained when chlorpromazine and verapamil were tested for their effects at both 10 and 0.1uM concentrations are shown in Table 32.

7) Effect of divalent ions on ^{45}calcium uptake.

Table 33 shows the results of experiments in which 1mM of the chlorides of calcium, strontium and barium were added to reaction mixtures containing a free calcium concentration of 2.5uM which was buffered only with ATP, in order to avoid the complexities of EGTA-divalent ion binding. Calcium was most effective in reducing the uptake of labelled calcium by PM and SR; it was not so effective in MITO. Strontium greatly reduced the ^{45}calcium uptake by all fractions; it was second best of the three ions in PM and SR and by far the best in MITO. Barium reduced ^{45}calcium uptake slightly in PM and MITO

TABLE 31

Effect of drugs at a concentration of 0.1mM on calcium uptake from solutions of 1uM calcium by subcellular fractions of rat skeletal muscle.

	Chlorpromazine	Propranolol	Verapamil
PM	50±21*	87±11	101±17
SR	43± 6*	97± 9	90± 9
MITO	7± 3*	121±28	94±25

Values expressed as mean ± s.e. of % of calcium uptake in the absence of added drug. Incubation time 30 seconds for PM and SR, 10 minutes for MITO. Number of preparations=4. * Denotes values significantly different from 100%.

TABLE 32

Effect of some drugs, at low concentration on calcium uptake by subcellular fractions of rat skeletal muscle.

	Chlorpromazine			Verapamil	
	10uM	0.1uM		10uM	0.1uM
PM	91±17	119±15		120±11	114±5
SR	86±15	87± 2*		100± 3	89±1*
MITO	72±12	105± 4		103± 1	100±1

Values expressed as mean ± s.e. of % of calcium uptake in the absence of added drug. Incubation times 30 seconds for PM and SR, 10 minutes for MITO. Number of preparations=3. * Denotes values significantly different from 100%.

TABLE 33

Effect of addition of divalent cation on
[45]calcium uptake by subcellular fractions of rat
skeletal muscle.

	Calcium chloride 1mM	Barium chloride 1mM	Strontium chloride 1mM
PM	2±1	83±12[1]	9±1
SR	4±1	102±4[1]	17±1
MITO	41±25	70±25[1]	9±7

Values expressed as mean ± s.e. of % of
[45]calcium uptake when no extra divalent cation
added," i.e. with free calcium concentration of
2.5uM. PM and SR incubated for 30 seconds, MITO
for 10 minutes. Number of preparations=3. All
values significantly different from 100% except
[1].

but was ineffective in SR.

8) Effect of lanthanum on ^{45}calcium uptake.

Attempts to determine the effect of pre-treatment of the PM and SR fractions with lanthanum prior to uptake studies met with the same problems as did the similar experiments conducted on the PM and ER fractions from rat myometrium. There was a loss of protein as a result of lanthanum pre-treatment. However, the results of ^{45}calcium uptake studies were somewhat more consistent; results are shown in Table 34. Uptake by both PM and SR fractions was greatly inhibited by pre-treatment with both 1 and 10mM lanthanum chloride.

TABLE 34

Effect of pre-incubation with lanthanum on
^{45}calcium uptake by subcellular fractions of rat
skeletal muscle.

	Lanthanum chloride 0.1mM	Lanthanum chloride 1mM	Lanthanum chloride 10mM
PM	62±10* (3)	9± 4 (3)*	9± 4 (3)*
SR	104±87 (3)	9± 2 (3)*	10± 3 (3)*

Values expressed as a % of the calcium uptake in
the control fractions which were not pre-
incubated with lanthanum. Incubation time 30
seconds. * Denotes values significantly
different from 100%. Numbers in brackets
represent number of preparations.

Chapter 5

DISCUSSION

In this chapter I will first discuss the results of experiments on rat myometrium and follow this with a discussion of the results of experiments on rat skeletal muscle.

A. Experiments on rat myometrium.

1) Isolation and characterization of subcellular fractions.

As judged by the activities of the plasma membrane markers used, the fraction which I considered to best represent plasma membrane is better than that described in the only previous study attempting to subfractionate and characterize rat myometrium (Kidwai, Radcliffe and Daniel, 1971). The specific activity of 5'-nucleotidase in the PM fraction (see Table 3) was almost twice that of the plasma membrane fraction isolated by Kidwai, Radcliffe and Daniel (1971) and represented a 14-fold increase in specific activity over that of the homogenate. Similarly there was an increase in the potassium-activated phosphatase activity of this plasma membrane fraction (see Table 4)

compared to that of Kidwai, Radcliffe and Daniel (1971), and this despite the fact that they used total values whilst I chose the ouabain-sensitive enzyme as a better marker. Potassium-activated, ouabain-sensitive phosphatase activity was hard to detect in the homogenate and a very substantial purifaction was obtained. As shown by Table 5 phosphodiesterase-I was increased almost 14-fold in specific activity from homogenate to the PM fraction. These findings together with the fact that there was a low activity of cytochrome-c oxidase in the PM fraction (see Table 6) provide good support for the claim that this fraction was much enriched in material of plasma membrane derivation, despite some doubts about the true distribution of one of the enzymes used (Song, Kappas and Bodansky, 1969).

It is unfortunate that the two endoplasmic reticular fractions obtained in this study had to be characterized to a large extent on negativities (i.e., low plasma membrane marker and low mitochondrial marker activities), however, the approach seems to be reasonably well justified. Certainly the electron micrograph shown in Plate 3 indicates that the fraction designated RER was substantially composed of material originating from the rough endoplasmic reticulum and this fits in well with the enzyme activities, although both cytochrome-c oxidase and azide-sensitive calcium uptake activities were higher than

is ideally desired. The isolation of the SER fraction which I acheived represents the first time that an endoplasmic reticular fraction of any kind (as opposed to microsomal) has been isolated from myometrium relatively free of mitochondrial contamination, whilst the RER fraction appears to be the first mainly rough endoplasmic reticular fraction isolated from a smooth muscle. As mentioned in Chapter 1 continuous density gradients seem to be preferred to discontinuous gradients (DePierre and Karnovsky 1973; and Wallach and Lin, 1973). In preliminary experiments I tried to utilize the continuous gradient separation technique of Kidwai, Radcliffe and Daniel (1971). The results from the use of this method were extremely disappointing in that they were extremely variable (perhaps due to a lack of uniformity of the gradients prepared on an ISCO density gradient former) and the separation of markers was, at best, poor. Slight modifications to the continuous gradient improved neither the reproducibility nor the separation obtained. The use of discontinuous gradients produced excellent reproducibility and a much better separation of markers than did the use of a continuous gradient (Kidwai, Radcliffe and Daniel, 1971). This improvement justifies the use of discontinuous gradients as opposed to the more rigourously correct continuous gradients.
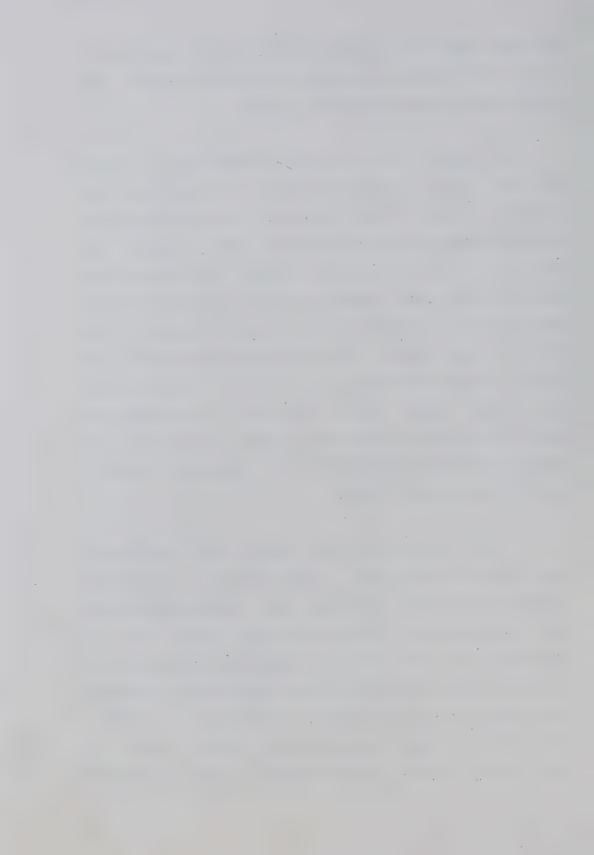
As discussed in Chapter 3 the mitochondrial fraction

obtained from rat myometrium showed all the properties of a truly mitochondrial fraction although it did have some plasma membrane marker activity present.

The purpose of this study of course was to enquire into the calcium uptake properties of material from different origins within the cell. Fractions as pure as could be reasonably obtained were of course desired for this purpose and it can be clearly seen that much improvement has been obtained, not only over the earlier microsomal and mitochondrial preparations (Batra, 1972, 1973; Batra and Daniel, 1970a; Batra and Timby, 1971; and Carsten, 1969, 1973a and b), in some cases of which little more than blind faith was used as an index of characterization, but also over the more recent attempt to purify subcellular fractions from the myometrium (Kidwai, Radcliffe and Daniel, 1971).

It can quite easily be argued that even greater purification is possible. Certainly the endoplasmic reticulum fractions contained some plasma membrane and some mitochondrially derived material; also, it is impossible to ascertain by the techniques used here just how much material derived from the endoplasmic reticulum was present in the PM fraction. As quick and as simple a purification procedure as possible was needed, with the hope that the calcium uptake mechanisms would be preserved

in as undamaged a state as possible, hence further
purifaction was not attempted.

2) ATP-dependent calcium uptake by the subcellular
   fractions.

a) Calcium transport and binding from solutions of 17uM
free calcium.

A great deal of information can be gained from the
results presented in Tables 7 and 9. One of the most
noticeable results of the study is that every fraction
investigated showed ATP-dependent calcium uptake and thus
the cellular structures from which these fractions are
derived may all contribute in appropriate circumstances to
myometrial calcium regulation. All fractions except the
mitochondria showed increased uptake in the presence of
oxalate; such an occurrence has been taken in other
systems to indicate the presence of a mechanism which
transports calcium from the external medium to the inside
of the vesicle (Hasselbach, 1964; Hurwitz et al. , 1973;
and Inesi, Ebashi and Watanabe, 1964), and is taken as
giving such an indication here. In all cases the
potentiation by oxalate was much smaller than that seen in
skeletal muscle, both by others (see Weber, 1966 for
references) and in this study. These results could be
taken to indicate that only a small amount of the net

uptake seen under normal conditions represents transport into the vesicular lumen. However, such an extrapoltion need not be justified. To show an effect with oxalate a system must fulfill several, not just one, requirements. Firstly, it must be vesicular (which seems to be the case for these fractions). Secondly, it must have a calcium transport mechanism. Thirdly, it must be permeable to oxalate so that this ion can get in and be precipitated with the higher concentrations of calcium (an unknown factor for these vesicles). Finally the vesicles must be relatively well sealed so that the intravesicular activity of calcium can reach a high enough order for the calcium oxalate solubility product to be exceeded (again an unknown factor for these vesicles). Thus an alternative explanation for the small oxalate effect could be that the fractions do show calcium transport but relatively few vesicles in each population have all the properties required for a massive oxalate effect. Indeed even in fragmented sarcoplasmic reticulum of skeletal muscle, Baskin and Deamer (1969) have presented evidence that suggests that only 15-25% of the vesicles demonstrate deposits of calcium oxalate when incubated in the appropriate loading medium and viewed electron microscopically using the negative staining technique. If this is true, then skeletal muscle sarcoplasmic reticulum preparations may not fulfill all the requirements to produce an oxalate effect throughout their entire

population of vesicles.

Also, since the oxalate effect seems to be greatest in the RER fraction followed by SER then PM, might it be a property of the endoplasmic reticulum or even only rough endoplasmic reticulum, and might its occurrence in the other fractions reflect contamination by RER? There is certainly a possibility this is so. The amount of endoplasmic reticulum in the PM fraction is not known nor is the amount of material derived from rough endoplasmic reticulum which contaminates the SER fraction. If the uptake of calcium by the PM fraction were to represent the manifestation of an outwardly directed pump, (i.e., if these vesicles are inside out), then one would expect transport into the vesicular lumen as well as binding to the membrane to take place. However, the smallness of the oxalate effect seen does not deny the presence of a calcium transport mechanism for the reasons outlined above. In general there was a good correlation between cytochrome-c oxidase activity and azide-sensitive ATP-dependent calcium uptake.

b) Uptake from solutions of low calcium concentration.

The crucial question is not whether these fractions can take up calcium from a solution of 17uM free calcium, but whether they can take up calcium in the range of 0.1-

1uM which, as outlined in Chapter 1, is probably the operating range of concentrations for contraction and relaxation. The results of the studies on the effect of calcium concentration on calcium uptake (expressed in Figures 3 and 4) make two very salient points: firstly the remarkable similarity in calcium uptake properties among PM, SER and RER, and hence presumably among the structures these are thought to be derived from; and secondly, the differences in such properties between PM, SER and RER on one hand and the mitochondrial fraction on the other.

The results presented here give a positive answer to the question posed by Devine, Somlyo and Somlyo (1973) as to whether rough endoplasmic reticulum can bind and transport calcium. The demonstration has come by a more direct technique than the search for electron-dense deposits within the reticulum after incubation of the muscle with strontium or barium, which was made by Devine, Somlyo and Somlyo (1973).

In the presence of ATP, the PM, SER and RER fractions have been shown able to accumulate calcium from solutions of 0.03 and 0.3uM. How reliable are the actual numerical values obtained at these concentrations? Firstly, the doubt about the absolute value of the free calcium concentrations which was mentioned in Chapter 2 must be borne in mind. Secondly, the technique that I used to

measure calcium uptake has been criticized (Carsten, 1969 and, 1973a; and Carvahlo, 1968) on the grounds that it measures calcium exchange and not calcium uptake; thus some of the calcium measured as being taken up by these fractions might possibly have been in exchange for calcium already present. Carsten (1973a) estimated that 25% of the calcium uptake by the microsomal fraction from pregnant bovine uterus as measured by the filtration technique was in fact exchange. Batra and Daniel (1971b), however, found no evidence for an ATP-dependent exchange of calcium by the microsomal fraction from rat myometrium. Thirdly, as seen from the influence of high sodium and high potassium concentrations upon calcium uptake by the PM, SER and RER fractions, the uptake exhibited may be somewhat reduced in the true environment of the cell below that measured here. Clearly the plasma membrane normally has an asymmetrical distribution of ions on either side of it and asymmetry of ionic distribution across these vesicles could not be obtained in these studies, so the effect of this asymmetry is unknown. This could be a very important factor particularly with respect to the possible involvement of a sodium-calcium exchange mechanism across the plasma membrane in regulation of intracellular calcium. The effect of sodium and potassium might be brought about via an action of these ions at a calcium binding site on a protein similar to calsequestrin. MacLennan and Wong (1971) have shown calcium binding to

calsequestrin to be reduced in the presence of high potassium.

Since it is not known what fraction of the vesicles in the PM, SER and RER fractions have their calcium binding or calcium transporting faces on the outside, (that is, facing the external medium), it is not possible to tell whether all or only a small fraction of the true capacity of the membranes to transport calcium is being measured. The net uptake of calcium measured at a given time is dependent upon both the rate of uptake and the rate of loss of calcium. If the vesicles were not completely sealed, or were in some other way rendered leaky by the isolation procedure, then the uptake measured would be an underestimate of the uptake capacity of the intact membranes.

At this point, having indicated the many uncertainties about the calcium uptake by the fractions I obtained here, it seems worthwhile to compare the activity of these fractions with those obtained from other studies on calcium accumulating mechanisms in uterus and other smooth muscles.

Other than pointing out that calcium uptake mechanisms exist in smooth muscles, the studies of Andersson, Lundholm and Mohme-Lundholm (1972), Batra

(1972), Carsten (1969, 1973a and b), Fitzpatrick et al. (1972) and Hurwitz et al. (1973) are of little value in indicating what processes might be important for relaxation, since no attempt was made to control the free calcium concentrations of the uptake media nor to set the concentrations in the range thought to be important for relaxation. Only three studies, those of Batra (1973) on human myometrium, Batra and Daniel (1971b) on rat myometrium and Baudouin-Legros and Meyer (1973), have made any attempt to measure uptake at calcium concentrations as low as 1uM. The most relevant study here of course is that of Batra and Daniel (1971a). Besides having succeeded in subdividing the microsomal fraction into plasma membrane, smooth endoplasmic reticulum and rough endoplasmic reticulum derived fractions, I also succeeded in preparing fractions which took up more calcium per mg protein than did the microsomal fraction of Batra and Daniel (1971a). Despite the differences in pH of the uptake reaction mixtures used (7.0 in my study and 7.2 in that of Batra and Daniel (1971a)), uptakes at 10 minutes from solutions of different calcium concentrations can be compared. Thus Batra and Daniel (1971a) could not detect uptake from solutions of 0.3uM whilst that from 0.6uM was 1.3 micromoles per g protein and that from 8.6uM was 5.5 micromoles per g protein. The PM, SER and RER fractions used in my study showed very similar uptakes to each other at low calcium concentrations. When the mean of uptake by

all three fractions was calculated from the data presented
in Figure 3 the following results were obtained: at 0.03uM
2.3 micromoles per g were taken up, at 0.3uM 4.7, at 1uM
11.6 and at 8uM 13.6 micromoles per g were accumulated
during incubation times of ten minutes. Although they did
not use any technique to buffer the calcium concentration
in the reaction mixture, Baudouin-Legros and Meyer (1973)
attempted to measure calcium uptake by a microsomal
fraction from rabbit aorta from a solution of 1uM added
calcium. It is difficult to say what the free calcium
concentration of the uptake-medium in this case was, since
it would be lowered below 1uM because of binding to ATP,
but there was probably some contaminating calcium present.
Thus it seems reasonably safe to assume that the calcium
concentration was not below 0.1uM. However, the calcium
uptake measured was well below 1 micromole per g protein.
Thus at low concentrations of calcium the PM, SER and RER
fractions used here are the most potent fractions of non-
mitochondrial origin yet isolated from smooth muscle.

The properties of the mitochondria prepared from rat
myometrium in this study as regards their ability to take
up calcium from low calcium solutions have already been
dealt with. Whilst it is difficult to extrapolate data
from a great variety of different experimental conditions,
the overall calcium uptake properties of these isolated
mitochondria appear to agree quite well with those of

mammalian origin investigated in several other studies, as regards rate, affinity for calcium and amounts bound (Carafoli and Lehninger, 1971; Drahota et al. , 1965; and Scarpa and Graziotti, 1973). Apart from those from the uterus, smooth muscle mitochondria have been ignored with respect to their ability to accumulate calcium. Batra and Daniel (1971a) discounted the possibility of a role for the mitochondria of the rat myometrium in the calcium regulation, of relaxation since calcium uptake by these organelles was severely inhibited in the presence of 125mM potassium. Batra (1972) observed a very much larger uptake of calcium by the mitochondrial fraction than by the microsomal fraction from human uterus and later reported (Batra, 1973) that these mitochondria could take up calcium from a concentration as low as 9.2nM. Since all my experiments on the effect of calcium concentration on calcium uptake were carried out in the presence of potassium ion, I cannot say whether this ion caused inhibition of calcium uptake by the mitochondria.

3) Mechanisms of the ATP-dependent calcium uptake processes.

As to the mechanism by which the fractions take up calcium, there appears to be little difference among PM, SER and RER but some differences between these fractions on one hand and the MITO fraction on the other.

a) Substrate specificity.

Tables 13 and 14 show that none of these fractions were able to increase their calcium uptake above ATP-independent uptake in the presence of a variety of other substrates. It seems that ATP is specifically required to support uptake by these fractions or that uptake supported by other substrates is very slow indeed.

b) Effect of univalent cations.

As far as the univalent ions are concerned not too much weight can be placed on the small differences seen in some cases, since uptakes from $0.3uM$ solutions are relatively small and differences would have to be quite large to be very significant. As mentioned earlier asymmetry of ionic distribution across these membranes may have resulted in an increase in calcium uptake in some fractions; asymmetry of ionic distribution could not be acheived.

c) Effect of drugs.

Batra and Daniel (1971a) showed that the sulphydryl inhibitor salyrgan blocked the accumulation of calcium by microsomes from rat myometrium just as it blocks calcium

transport by skeletal muscle microsomes. When the effects of chlorpromazine, propranolol and verapamil, which have been shown to inhibit calcium uptake by skeletal muscle fractions (Balzer, 1972; and Balzer, Makinose and Hasselbach, 1968) and D600, which has been shown to inhibit calcium uptake by the microsomal fraction from heart (Entman et al. , 1972), were tested on PM and SER fractions from rat myometrium, they were found to inhibit calcium uptake in this system also (results are shown in Table 17). Generally the SER fraction was more inhibited than was the PM fraction. The inhibitions produced by the drugs on calcium uptake by both fractions agree quite closely with the results obtained from the other muscles except that little or no inhibition was produced by 0.1mM verapamil in the uterus fractions, whereas both heart and skeletal muscle fractions were inhibited to some extent. The conditions used by Balzer (1972) and Balzer, Makinose and Hasselbach (1968) were very similar to those which I used except that oxalate was present in the reaction mixture. The studies on the heart fraction (Entman et al. , 1972) used different conditions but did examine uptake in the absence of oxalate. Thus as affected by these drugs, the calcium uptake mechanism of the PM and SER fractions appears to be similar to that of skeletal and cardiac muscle. Balzer (1972) and Balzer, Makinose and Hasselbach (1968) found propranolol, chlorpromazine and verapamil to inhibit the calcium-dependent ATPase of the

sarcoplasmic reticulum of skeletal muscle. Entman et al.
(1972) however, reported that they could find no
inhibition of cardiac muscle microsomal calcium-ATPase by
D600 or verapamil. I did not test the effect of these
drugs on ATPase activity of uterine fractions. It should
be noted that chlorpromazine (Hodgson and Daniel, 1973;
and Frankenheim and Shibata, 1968), D600 (Fleckenstein et
al., 1971) and verapamil (Fleckenstein et al., 1971; and
Haeusler, 1971), have all been shown to inhibit the
contractility of smooth muscles including that of the
uterus. An inhibition of the mechanisms responsible for
lowering the cytoplasmic calcium concentration does not
seem to be compatible with an inhibition of contractility,
since if calcium removal stopped whilst all other
conditions remained the same, the cytoplasmic calcium
concentration would increase and the muscle would
contract. However, the studies mentioned above all showed
inhibitory effects of these drugs on smooth muscles at
concentrations which were one or two, and in some cases,
three orders of magnitude lower than those used here. The
question arose as to whether lower concentrations of these
drugs might promote calcium uptake. Table 18 shows that
no such increase in calcium uptake was found when
chlorpromazine and verapamil were tested at 0.1 and 10uM
on the pre-steady state calcium uptake by the uterus
fractions. Thus such an action does not appear to account
for the inhibitory properties of these drugs on

contractility. It has been suggested that chlorpromazine (Hodgson and Daniel, 1973; and Frankenheim and Shibata, 1968), D600 (Fleckenstein et al. , 1969; and Mayer, VanBreemen and Casteels, 1972), and verapamil (Fleckenstein et al. , 1969; and Haeusler, 1971) produce their inhibitory effects by blockade of the transmembrane calcium fluxes associated with excitation-contraction coupling. If this is true then these fluxes must be either more sensitive or more accessible to the drugs in the intact tissue than are the calcium-pumping mechanisms. It is noteworthy that calcium uptake by the mitochondrial fraction from rat myometrium was also sensitive to the drugs studied; in fact it was the most sensitive of the three uterine fractions studied was and also more sensitive than the systems studied by Balzer (1972), Balzer, Makinose and Hasselbach, (1968), and Entman et al. (1972).

d) Effect of divalent cations.

The effects of barium and strontium ions in antagonising the uptake of labelled calcium by the fractions from rat myometrium pointed to no obvious differences amongst PM, SER and RER. Batra and Daniel (1971a) found 1mM strontium to completely inhibit calcium uptake, and found 1mM calcium to greatly supress the uptake of labelled calcium.

The addition of extra cold calcium is of course a necessary control experiment for the effects of both barium and strontium upon the uptake of labelled calcium. In my experiments, the results of which are shown in Table 19, the addition of extra calcium greatly reduced the amount of label taken up by all fractions. Such a result is to be expected since under control conditions 15uM calcium was present, whereas in the case of extra calcium addition a total of 1015uM calcium was present. The specific activity of the label was reduced approximately 70 times by the addition of extra calcium.

If the reduction of the specific activity of the calcium after extra calcium addition were the only factor affecting the amount of radioactivity taken up, then the calcium addition should have resulted in a reduction in the amount of label taken up to about 1.5% of the control value. However, the addition of extra calcium would change the free calcium concentration in the medium from 2.5 to approximately 150uM. Now as is shown by Figures 3 and 4 there are complex relationships between the free calcium concentration in the medium and the amount of calcium taken up by PM, SER, RER, and MITO. The results shown in Table 19 indicate that the change in free calcium concentration resulted in an increase in net calcium uptake of approximately 3-fold in the cases of SER, RER

and MITO and in little change in uptake by PM.

In the case where extra strontium was added, both the amount of calcium bound to ATP and the amount of free divalent ion will be different from the respective values in the case of addition of extra calcium, since ATP discriminates between calcium and strontium (having different binding constants for the two ions, Naninga, 1961). Unfortunately the binding constants for strontium and for barium to ATP have not been determined by a method compatible with that used to determine the binding constants for calcium and magnesium to ATP used throughout this thesis (Godt, 1974). Thus, it is not possible to estimate the free and bound concentrations of these divalent ions in this case.

If the uptake mechanisms could not distinguish between calcium and strontium, then the specific activity seen by the mechanisms would be represented by the amount of free radioactivity in the medium divided by the total free divalent ion in the medium. Since the binding constant for strontium to ATP is less than the binding constant for calcium to ATP (Naninga, 1961), the specific activity would be higher than in the case of addition of extra calcium. Uptake of label under such circumstances would be higher than the uptake of label in the case where extra calcium was added (the increase is likely to be 2-

to 4-fold, but cannot be accurately determined without the correct constants).

Alternatively if the uptake mechanism did not recognize strontium at all the specific activity would remain unchanged, although the free calcium concentration would be slightly elevated. In this latter case, uptake of label should be 100% of control or slightly higher. Clearly, this is not so for the case of extra strontium addition to all four fractions from rat myometrium. Thus it appears that strontium can mimic calcium at some step in the overall uptake mechanism in all these fractions, but whether or not it does so by being indistinguishable from calcium cannot be determined from the data available.

Similar arguements to those used in the case of extra strontium addition are applicable in the case of extra barium addition. In SER barium is not recognized by the uptake mechanism, whereas in PM, RER and MITO it is, but the precise nature of the interaction cannot be determined.

e) Effect of lanthanum.

It is unfortunate that a really satisfactory method could not be found to investigate the effects of the lanthanum ion on calcium uptake since it may have revealed

some differences amongst PM, SER and RER. Since the report of VanBreemen et al. (1972) on the use of lanthanum as a tool for the study of transmembrane calcium fluxes concerned with excitation-contraction coupling in aorta, attempts have been made to use lanthanum to study the same phenomena in rat uterus (Hodgson and Daniel, 1973; and Marshall and Kroeger, 1973). Amongst the properties of lanthanum necessary for its use in this way, is a total blockade of transmembrane calcium fluxes so that calcium can neither enter nor leave the muscle cell and thus an estimation of the cellular contents of calcium can be made at various stages of excitation. However, Hodgson and Daniel (1973) showed that the lanthanum technique was not valid for rat myometrium since 2mM lanthanum did not completely inhibit calcium uptake into such tissues. The experiments which I carried out were an attempt to see if the other half of the assumption, namely a total inhibition, of calcium extrusion was equally invalid. No firm conclusions about this can be drawn. Obviously the validity of the assumptions concerning the lanthanum technique as applied to myometrium are in some cases diffucult to test and in other cases not valid (see also Hodgson, Kidwai and Daniel, 1972); thus interpretation of results obtained by its use should be reserved.

f) Calcium-ATPase.

All fractions showed a consistent increase in ATP hydrolysis upon addition of calcium. However, in most cases this was quite a small difference. Since the numerical values of the extra-splitting detected were small as compared to the basal ATPase, in the absence of calcium, the significance of the differences obtained for some of the fractions is doubtful. However, even if the extra-splitting seen is a real phenomenon, the question remains whether it represents activity of a calcium-ATPase responsible for calcium transport. If values are taken from Figure 3 it is found that under similar conditions to those used to measure ATPase activity the PM, SER, RER and MITO fractions accumulated 7.8 ± 0.8, 10.4 ± 2.4, 7.6 ± 1.1 and 23.9 ± 7.8 micromoles of calcium per g protein per ten minutes, respectively. If the values given in Table 16, where the units are micromoles Pi released per mg per 10 minutes, are first multiplied by 1,000 to convert them to micromoles Pi released per g protein per 10 minutes and these values are then divided into the values given above, the apparent number of moles of calcium transported per mole of inorganic phosphate liberated will be given. The values are 0.00065, 0.00208, 0.00095 and 0.01195 moles of calcium transported per mole of inorganic phosphate released by PM, SER, RER and MITO, respectively. Batra and Daniel (1971) could find no extra-splitting due to

added calcium in their microsomal preparation from rat myometrium. They argued that if there was indeed a calcium-ATPase that was responsible for calcium transport, and if the stoichiometry was the same as that reported for skeletal muscle (namely 2 moles of calcium taken up per mole of phosphate released), then the extra ATPase activity would not be detectable by the technique of measuring inorganic phosphate liberated. Carsten (1969) claimed to find extra-splitting of ATP due to added calcium by the microsomal fraction from cow uterus although the assay conditions which she used were not consistent, EGTA being present only in the reaction mixture measuring basal ATPase and not being present when calcium was added. Carsten (1969) did not attempt to relate ATP hydrolysis to calcium uptake. The remarks of Batra and Daniel (1971) are correct for the fractions under study here. If, for example, calcium transport in the PM fraction were coupled to ATP hydrolysis such that two calcium ions were transported whilst one molecule of inorganic phosphate was liberated, then the extra ATPase activity of the PM fraction would be expected to be about 0.00039 micromoles Pi released per mg protein per 10 minutes. Such values are certainly outside the limits of detection by the technique used.

What then do the values obtained here represent? They suggest that the extra hydrolysis of ATP induced by

the presence of calcium might not be tightly coupled to calcium accumulation. For instance, calcium uptake might be dependent upon ATP hydrolysis; however if the vesicles are very leaky such that much more calcium is transported over the measured time than the net accumulation measured at the end of that time indicates, then the stoichiometry calculated from calcium uptake and phosphaterelease under these conditions will be in error. If an increase in extra ATP hydrolysis due to leak of transported calcium is the explanation, and the only explanation, then the PM and SER fractions, to take these as examples, would have transported 24,000 and 16,000 micromoles of calcium per g of protein in ten minutes respectively, provided 2 moles of calcium were transported per mole of ATP hydrolysed. The presence of other, non transporting, ATPases which are stimulated by calcium might also explain part of these observations. These experiments cannot rule out the presence of such ATPases.

4) ATP-independent calcium uptake.

The results shown in Figure 3 indicate that at low calcium concentrations, a large fraction of the total calcium taken up by the vesicular fractions in the presence of ATP can be taken up in the absence of ATP. The calcium taken up by mitochondria at 0.03 and 0.3uM free calcium concentrations was almost exactly the same in

the absence as in the presence of ATP. This ATP-independent uptake might represent calcium exchange.

5) Summary of work on rat myometrium.

Four major subcellular fractions have been obtained and designated PM, SER, RER and MITO; they are considerably enriched in material derived from plasma membrane, smooth endoplasmic reticulum, rough endoplasmic reticulum and mitochondria, respectively. All fractions can accumulate calcium in the presence of ATP and in some cases there is a suggestion that calcium is being transported, as evidenced by a potentiating effect of oxalate. The fractions enriched in material from the plasma membrane, the smooth endoplasmic reticulum and the rough endoplasmic reticulum can accumulate calcium from solutions where the free concentration of calcium is 0.03 and 0.3uM, while the mitochondrially enriched fraction cannot. Since nothing is known of the orientation of the vesicles in each fraction nor of the amount of vesicles which are sealed, it is impossible to say how much of an underestimate of the true binding capabilities of the fractions the values measured here are.

As judged by the effect of drugs the calcium uptake mechanism of the PM and SER fractions appears similar to those of skeletal and cardiac muscle microsomal fractions.

PM, SER and RER fractions differ from skeletal muscle (see later) in specifically requiring ATP as opposed to several other substrates in order to accumulate calcium. Calcium uptake by the PM, SER and RER fractions does not differ in its sensitivity to blockade by strontium and barium ions, although that by MITO is somewhat more sensitive to blockade by strontium than that by PM, SER and RER. The MITO fraction has a greater sensitivity to drugs which reduce calcium uptake than any of the other systems studied. It too requires ATP as the substrate for calcium uptake, but in the presence of ATP, addition of succinate increases the amount of calcium that can be taken up. In no case did several drugs which inhibit uterine contractility produce an increase in calcium uptake by any of the fractions tested. All fractions showed an increase in ATP hydrolysis when calcium was present as opposed to when it was absent, but this difference was only significant in the PM and RER fractions. This could in part represent a calcium-stimulated, transport-linked ATPase but it has not been conclusively shown to do so.

B. Experiments on rat skeletal muscle.

1) Isolation and characterization of subcellular fractions.

The fraction which I considered to best represent plasma membrane was certainly enriched in the plasma membrane marker enzymes when compared to the other fractions isolated. 5'-Nucleotidase specific activity was enriched 4 times in the PM fraction as compared to the homogenate (Table 23), whilst the ouabain-sensitive, sodium+ potassium-activated ATPase specific activity was enriched by a factor of 3 in the PM fraction as compared to the SR fraction (ouabain sensitivity being undetectable in the homogenate). As shown in Table 24 the PM fraction contained some, though not a large amount, of cytochrome-c oxidase activity, but calcium uptake by this fraction was not significantly affected by the presence of 0.5mM sodium azide, indicating that mitochondrial contamination was not substantial. The fraction I consider to best represent sarcoplasmic reticulum was lower in the plasma membrane marker specific activities than was the PM fraction, and showed calcium uptake properties which have normally been attributed to material derived from the sarcoplasmic reticulum. Cytochrome-c oxidase activity could be detected in the SR fraction but again the effect of azide on calcium uptake by this fraction was not significant.

These results can be compared to those of Kidwai et al. (1973), who used a very similar technique to subfractionate rat skeletal muscle. The differences are quite striking. First of all Kidwai et al. (1973) reported that they could find no beta-glycerol-phosphatase activities in the fractions which they obtained from the sucrose density gradient whilst I found this activity in all fractions from the sucrose density gradient. The processes which I used up to the sucrose gradient step were almost identical to those used by Kidwai et al. (1973) and I cannot explain the discrepency between our results, unless it is related to the difference in the weights and thus the ages of rats used, 160-200g in my case and 200-250g in the case of Kidwai et al. (1973).

The activity of 5'-nucleotidase in the homogenate of skeletal muscle was 3 times higher in my experiments than in those reported by Kidwai et al. (1973) and this despite the fact that my values are corrected for beta-glycerol-phosphatase whilst those of Kidwai et al. are not. Again, I can offer no explanation for this discrepency except the age of the animals. The values of 5'-nucleotidase activity in the plasma membrane fraction are quite close, mine being slightly lower, however, because of the differences in homogenate activity. I was only able to obtain a 4-fold purifaction whilst Kidwai et

al. (1973) obtained a 17-fold purifaction of 5'-nucleotidase from homogenate to plasma membrane fraction. The specific activity of 5'-nucleotidase in the SR fraction which I prepared was half of that in the F2 (sarcoplasmic reticulum) fraction of Kidwai et al. (1973). The ratio of specific activity of 5'-nucleotidase in plasma membrane fraction to sarcoplasmic reticulum fraction was 4:1 in my experiments and 2.1:1 in those of Kidwai et al. (1973).

The reverse situation is true for the specific activity of the ouabain-sensitive, sodium+ potassium-activated ATPase. The PM fraction obtained in my studies had an activity of this enzyme which was almost five times greater than that of the F1 (plasma membrane) fraction of Kidwai et al. (1973). Kidwai et al. (1973) could detect no ouabain sensitivity in their F2 fraction whereas I found considerable (nearly one third of the PM specific activity of this enzyme) in the SR fraction. In neither case could inhibition by ouabain be detected in the homogenate.

As judged by the activity of 5'-nucleotidase, I did not obtain as good a purifaction of the plasma membrane as did Kidwai et al. (1973). However, my sarcoplasmic reticulum fraction was less contaminated by material originating from the plasma membrane than was theirs. As

judged by the activity of the ouabain-sensitive, sodium+ potassium-activated ATPase, I obtained greater purifaction of the plasma membrane fraction than did Kidwai et al (1973), although there was more contamination of the sarcoplasmic reticulum fraction by material from the plasma membrane in my case than in theirs.
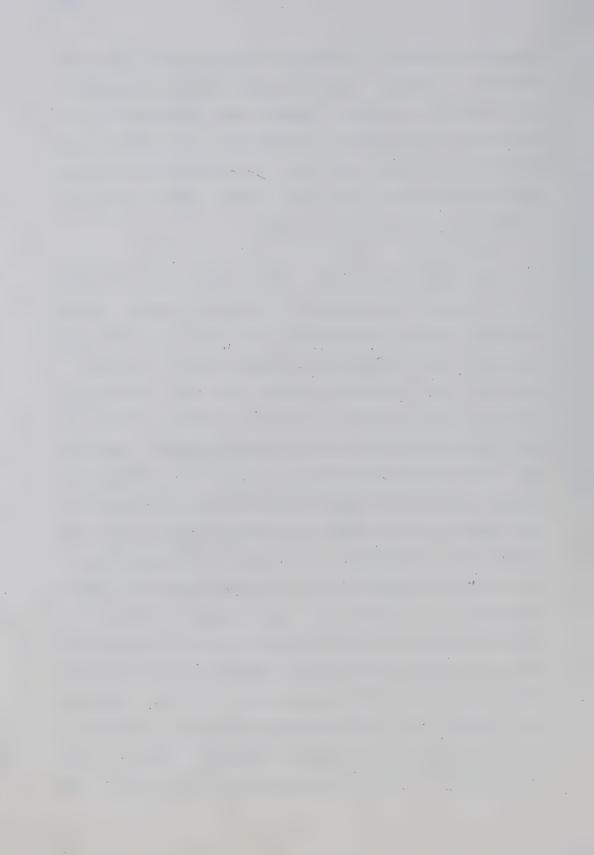
I found some cytochrome-c oxidase activity in all the subcellular fractions which I examined. Kidwai et al. (1973) reported that this enzyme was exclusive to one fraction, F3 (mitochondria), provided that homogenization was done carefully. My results were unable to substantiate this finding.

Another system which my results can be compared to is that of the skeletal muscle plasma membrane fraction, (termed by the authors, sarcolemma) prepared by Severson, Drummond and Sulakhe (1972) and later used by Sulakhe, Drummond and Ng (1973) to investigate the calcium uptake properties of skeletal muscle plasma membrane. Direct comparisons are not possible since Severson, Drummond and Sulakhe (1972) used rabbit skeletal muscle and assayed some different marker enzymes. However, they found no cytochrome-c oxidase activity in their sarcolemma preparation. They found ouabain-sensitive, sodium, potassium-stimulated ATPase to be present in this preparation and obtained a 15-fold increase in the
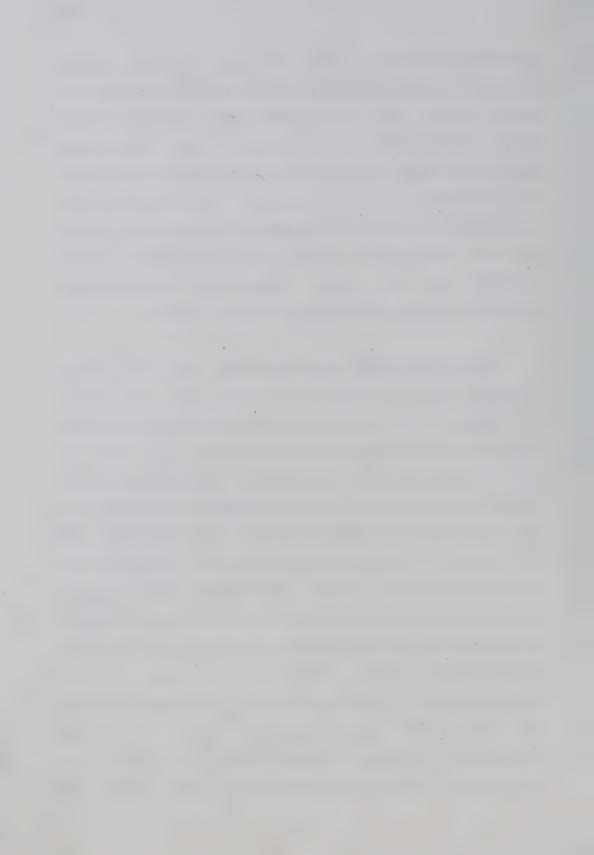
specific activity of adenylate cyclase in the sarcolemma fraction as compared to the muscle whole homogenate. Unfortunately Severson, Drummond and Sulakhe (1972) did not isolate sarcolemmal, mitochondrial and sarcoplasmic reticular fractions from the same muscle, and so the ratios of activities of the various enzyme activities amongst such fractions are unknown.

Of course any study which seeks to determine the calcium uptake properties of skeletal muscle plasma membrane should be capable of indicating with some accuracy, the possible amount of contamination of such a fraction by material derived from the sarcoplasmic reticulum, so that calcium uptake by material derived from the plasma membrane can be accurately assessed. Kidwai et al. (1973) used an estimate of protein biosynthesis by their fractions in order to estimate the contamination of the plasma membrane fraction by material derived from the sarcoplasmic reticulum. I did not use this technique in my experiments because there is no certainty that protein biosynthetic activity of the internal membranes of skeletal muscle is closely associated with or inseparable from the specialized calcium uptake properties of these membranes. Such a view was also put forth by Martonosi and Halpin (1972) who studied the turnover of proteins in the sarcoplasmic reticulum of skeletal muscle. They stated, ''The site of synthesis of the protein and

phospholipid components of sarcoplasmic reticulum within the muscle cell is unknown. The observed incorporation of amino acids into..... skeletal muscle microsomes is not sufficiently defined to conclude that sarcoplasmic reticulum membranes are involved in protein synthesis.'' This statement is still true today, and before protein biosynthesis is used as a marker, its validity as a marker should be demonstrated. Also it has been reported (Kidwai , 1973) that the plasma membrane of skeletal muscle possesses protein biosynthetic activity per se.

Several different techniques were used by Sulakhe, Drummond and Ng (1973) to demonstrate that there was no contamination of their sarcolemma by material derived from the sarcoplasmic reticulum; unfortunately, most of them were invalid. Thus the sarcoplasmic reticulum preparation which they compared to their sarcolemma was prepared by an entirely different technique than was the sarcolemma, and no attempts at characterization whatsoever were made on this former fraction. Hence the possible contamination of the so called sarcoplasmic reticulum fraction of Sulakhe, Drummond and Ng (1973) by material derived either from the plasma membrane or from mitochondria is unknown. Sulakhe, Drummond and Ng (1973) prepared their sarcolemmal fraction by treating the muscle homogenate with both lithium bromide and potassium bromide solutions, whilst their isolation of sarcoplasmic reticulum did not involve such
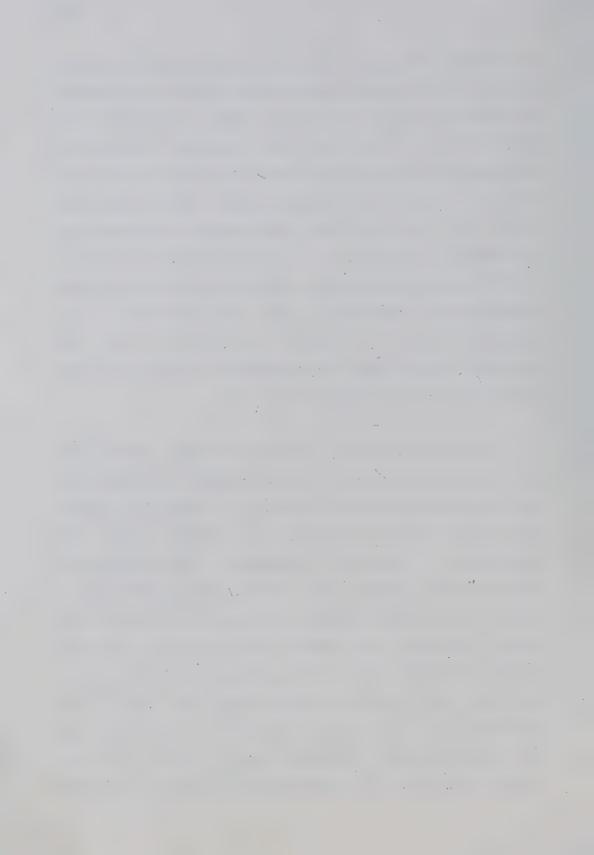
treatment. In the light of the results of Repke and Katz (1969) it is of little surprise that Sulakhe, Drummond and Ng (1973) found their sarcoplasmic reticulum fraction to be less active in taking up calcium after it too had been treated with both lithium bromide and potassium bromide solutions. However, Sulakhe, Drummond and Ng (1973) took such results as an indication that their sarcolemma was not contaminated by material from the sarcoplasmic reticulum. This is an invalid conclusion until it can be shown that these salts do not reduce the calcium uptake ability of the sarcolemmal preparations; this demonstration has not been made.

After having shown that the salt extraction inactivates calcium uptake by the sarcoplasmic reticulum, Sulakhe, Drummond and Ng (1973) added an amount of sarcoplasmic reticulum fraction to a muscle homogenate from which they then isolated sarcolemma by the usual procedure. Since they found only a small increase in the specific activity of calcium uptake in the sarcolemma thus isolated, Sulakhe, Drummond and Ng (1973) claimed no contamination by sarcoplasmic reticulum. They state that since sarcoplasmic reticulum had a much higher calcium binding activity than sarcolemma, the absence of contamination was proved. They seemed to be unaware that the sarcoplasmic reticulum only had a higher ability to accumulate calcium when not treated with lithium bromide

and potassium bromide. The yields which Sulakhe, Drummond and Ng (1973) reported for sarcolemma when it was isolated from muscle homogenate or from muscle homogenate plus sarcoplasmic reticulum are almost identical. However, it is unclear whether the yields were calculated on the basis of mg per g of total starting material (i.e., that from which both sarcolemma and sarcoplasmic reticulum were isolated) or of mg per g of tissue which was used to isolate sarcolemma (and thus ignorning the amount of added sarcoplasmic reticulum); this of course is a very important point to consider in respect to how much material derived from the sarcoplasmic reticulum can find its way into the sarcolemmal fraction.

These tests, as well as those in which sarcoplasmic reticulum was added to a final sarcolemma preparation and the mixture sedimented at 2,000xg for 10 mins then assayed for calcium uptake ability, are invalid since the sarcoplasmic reticulum preparation was prepared by discarding all material which sedimented at 8,000xg for 20 minutes whilst the sarcolemma was prepared from material which sedimented at 2,000xg for 10 minutes. Sulakhe, Drummond and Ng's (1973) findings demonstrate nothing more than the fact that the principles of differential centrifugation are valid. Several other differences observed by Sulakhe, Drummond and Ng (1973) might be better explained by the differential effects of treatment

with bromide salts than by differences between plasma membrane and sarcoplasmic reticulum; this possibility was not tested.
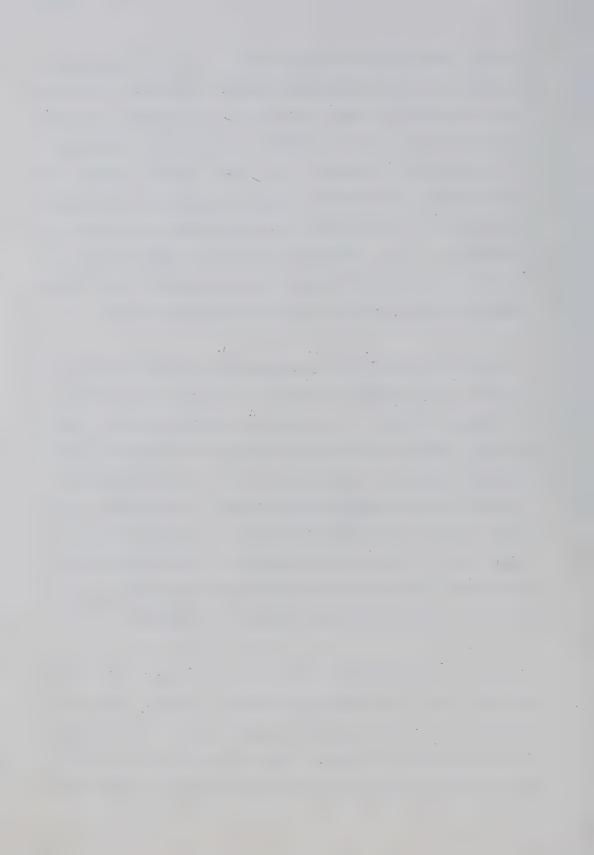
Sulakhe, Drummond and Ng (1973) found that if a muscle mince was incubated with phospholipase C and then both sarcoplasmic reticulum and sarcolemma were isolated from this and a control mince, calcium uptake by the treated sarcolemma preparation was greatly reduced when compared to the control, whereas calcium uptake by the treated sarcoplasmic reticulum preparation was only very slightly reduced as compared to the control. Sulakhe, Drummond and Ng (1973) took these results as a strong indication that their sarcolemma preparation contained material of surface origin since they assumed that phospholipase C was unable to penetrate into the cell and inactivate the sarcoplasmic reticulum. However, in a fine muscle mince such as the one these authors used to isolate their fractions from, one would expect a large number of cells to be broken open in such a way that phospholipase C could penetrate and thus affect intracellular membranes. The reproducibility of such experiments is not certain; Sulakhe, Drummond and Ng (1973) report the result from only one. Attempts to repeat the phospholipase C treatment experiments of Sulakhe, Drummond and Ng (1973) on rat skeletal muscle using the same isolation technique that I have used (Janis, 1973), resulted in gross changes

in the sedimentation properties of all subcellular
fractions including the mitochondria, indicating that the
action of phospholipase C could not be restricted to the
surface membrane. These results may indicate a difference
in sensitivity between rat and rabbit muscle to
phospholipase C treatment. However, Sulakhe, Drummond and
Ng (1973) did not show that the effects they saw were not
produced by an interaction between phospholipase C
treatment and lithium bromide treatment, rather than being
produced directly by the action of phospholipase C.

Thus no workers have unequivocally demonstrated the
absence, or indeed indicated the extent of the presence,
of material able to accumulate calcium derived from
skeletal muscle sarcoplasmic reticulum in skeletal muscle
membrane fractions. This is true of my experiments also.
I could find no technique based on well established facts
which would enable me to do this. I therefore relied on
being able to find some differences in the calcium uptake
properties of the PM and SR fractions that might at least
give some indication of two separate processes.

All the data which I obtained concerning the MITO
fraction from rat skeletal muscle indicate that this
fraction was indeed composed of material that was mainly
of mitochondrial origin. Thus the electron microscopic
appearance was that of a mitochondrial fraction (see Plate

9), and the fraction had a high specific activity of cytochrome-c oxidase and an ATP-dependent calcium uptake mechanism that was almost totally inhibited by 0.5mM azide.
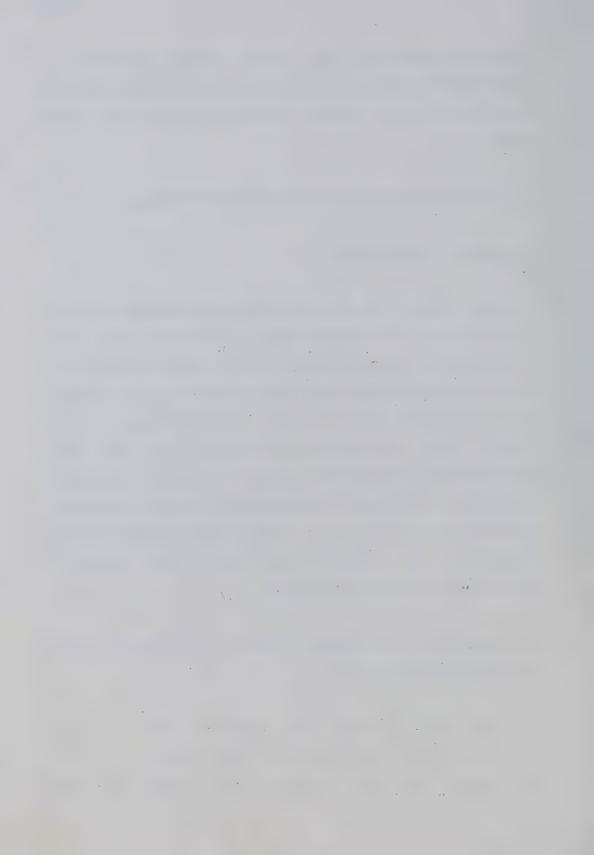
2. Calcium uptake by the subcellular fractions.

a) General observations.

The results of preliminary calcium uptake studies, shown in Table 25, revealed that all fractions could take up calcium in the presence of ATP, the MITO fraction being most active and being completely supressed in the presence of 0.5mM azide. Both PM and SR took up calcium, SR being almost twice as active whilst uptake by both these fractions was potentiated by oxalate, the actual numerical values for uptake in the presence of oxalate being almost identical in PM as in SR. Thus calcium transport was apparently more effective in PM than in SR. Neither PM nor SR were inhibited by azide.

b) Comparison of the calcium uptake data obtained with those obtained by others.

How do the values for calcium uptake by the SR fraction compare with those from other studies? It must be first of all understood that there is little

consistency in the techniques used from one laboratory to
another in order to measure calcium uptake; however, in
his review Inesi (1972) indicates that the maximal amount
of calcium accumulated by sarcoplasmic reticulum fractions
from   rabbit   skeletal   muscle   in   the   absence of
precipitation by agents such as oxalate is 110-220
micromoles per mg protein.   These values are 4-8 times
higher than those which I obtained for the SR fraction.
For the case where oxalate was present in the reaction
mixture, the conditions which I used were very similar to
those of Balzer, Makinose and Hasselbach (1968), who found
a mean value of 1,480 micromoles of calcium taken up per
minute per g protein at 20°C; in my experiments the SR
took up 1,417.7 micromoles calcium per g protein in one
minute at 37°C. The large discrepancy between the values
obtained by others with respect to uptake in the absence
of oxalate compared to my results, and the reasonably good
agreement seen in the case of uptake in the presence of
oxalate, might indicate that a greater part of the net
calcium uptake by the vesicles which I prepared was due to
calcium transport, or that less calcium could be bound to
the membrane of the vesicles that I prepared, or that the
vesicles which I prepared were more leaky to ATP-
dependently accumulated calcium that the vesicles prepared
by others.

Several   factors   could   account   for   an   increased

leakiness of the vesicles I prepared compared to those of others. Firstly, vesicles prepared from rat muscle might be naturally more permeable than those from rabbit; secondly, the prolonged sucrose gradient treatment that I used might have rendered the vesicles more leaky; and thirdly, the fact that I measured uptake at 37°C whereas others normally use 20-25°C might be responsible for the lower net uptake in the absence of oxalate, since it has been shown (Duggan and Martonosi, 1970) that vesicles of sarcoplasmic reticulum release more and more of the calcium accumulated at 25°C when the temperature of the medium into which efflux is allowed is increased stepwise from 4 to 40°C.

With the aid of a recent publication (Huxtable and Bressler, 1973) it ought to be possible to determine whether the combination of the above three factors is responsible for the discrepancy, since they prepared a sarcoplasmic reticulum fraction from rat skeletal muscle by subjecting it to a two hour sucrose density gradient centrifugation and they measured calcium uptake at 37°C. If their units can be taken seriously then the uptake values obtained by Huxtable and Bressler (1973) in the absence of oxalate (200-300 micromoles per mg protein in one minute from a solution of 50uM added calcium in the presence of 4mM ATP) are ten times greater than those which I obtained for the SR fraction (20-30 micromoles per

mg protein in one minute, from solutions of 1-100uM free calcium). However since in two of their graphs (Figures 1 and 3) Huxtable and Bressler show values for uptake in the presence of oxalate which are alternately 10 times greater and 10 times smaller (almost exactly, in both cases) than the values which I obtained (and since they appeared to use exactly the same conditions in the two cases), it seems that they were somewhat confused about units. The closeness of the numbers for uptake in the two cases tempt speculation; however, if Huxtable and Bressler (1973) did give the correct units for calcium uptake by their sarcoplasmic reticulum in the absence of oxalate, I can offer no explanation as to why my values are ten times smaller.

A feature which the results of Huxtable and Bressler (1973) have (both PM and SR fractions) in common with mine is the fall off in net accumulated calcium which occurs in the absence of oxalate and after one minute of incubation. Such a phenomemon has not been extensively reported for the rabbit sarcoplasmic reticulum. Since it did not occur either in my experiments or in those of Huxtable and Bressler (1973) when oxalate was present it may result from an increased leakiness of the rat skeletal muscle membranes following accumulation of calcium.

From Figure 3, calcium uptake by SR from a 1uM

solution was 18.7 micromoles per g in 30 seconds at 37ºC, whilst Table 22 shows that SR took up 23.7 micromoles per g in 20 seconds at 20ºC. ATP-dependent uptake should be a highly temperature-sensitive process (Inesi, 1972). If calcium uptake were the only limiting process in net calcium accumulation one would expect the values obtained at 37ºC to be much higher than those at 20ºC. However, since net accumulation is governed by differences in the rate of uptake and in the rate of efflux of calcium, it seems probable that efflux is greater at 37ºC than at lower temperatures.

3) Evidence for separate calcium uptake mechanisms in the plasma membrane, sarcoplasmic reticulum and mitochondria of rat skeletal muscle.

In subsequent experiments on calcium uptake the PM fraction behaved merely like a less active counterpart to the SR fraction. Thus both fractions showed similar time courses of calcium uptake from low and high calcium solutions and in the presence of oxalate; they showed the same response of uptake to different calcium concentrations (Figure 6), to drugs (Tables 32 and 33) and to divalent ions (Table 33). In most of these cases there were marked differences between the PM and SR fractions on one hand and the MITO fraction on the other.

The SR showed the well established property (Inesi, 1972; and Weber, 1966) of being able to accumulate calcium from low calcium concentration solutions; this property was mirrored by the PM fraction but not by the MITO fraction. Further to this the results of experiments shown in Table 37, in which uptake measurements were made at 20ºC after 20 seconds of incubation, indicate a wide discrepancy in the rates of uptake between PM and SR on one hand and MITO on the other, which suggests that PM and SR have a much more important role in calcium uptake and relaxation than does MITO.

a) Substrate specificity

In Chapter 1 it was pointed out that GTP, ITP, acetyl phosphate and para-nitrophenyl phosphate have all been shown to promote calcium uptake by skeletal muscle sarcoplasmic reticulum, though uptake in these cases had a slower rate and the mechanism showed a lower affinity for calcium than it did in the presence of ATP. The results shown in Table 28 confirm that all these substrates could support calcium uptake by both PM and SR fractions. The fact that net calcium uptake was the same for ATP, GTP and ITP under the conditions used agrees with the results of Martonosi and Feretos (1964), which indicate that at high concentrations of free calcium (like the ones which I used) the steady-state accumulation of calcium is the same
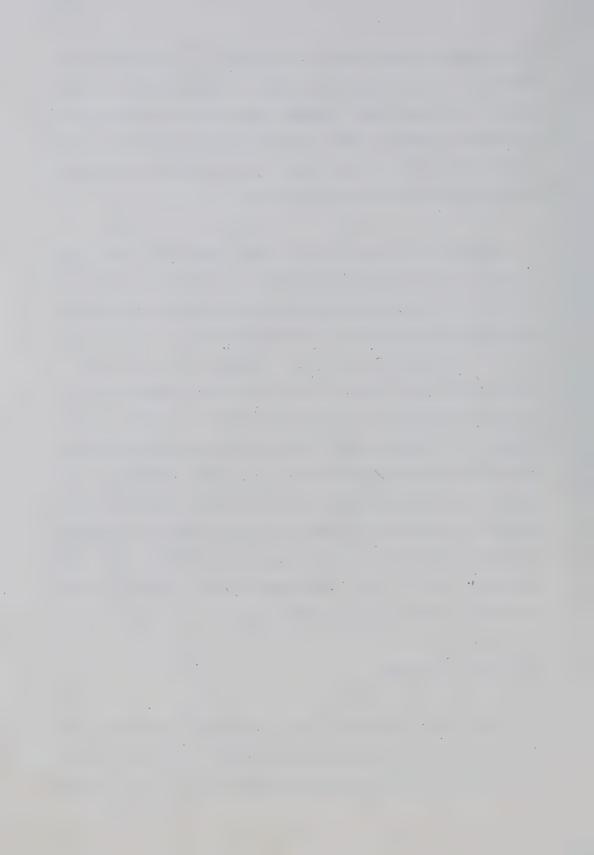
for several substrates. This result is probably brought about by the fact that the free intravesicular calcium exerts a controlling effect upon the rate of substrate hydrolysis (Weber, 1971). This feedback mechanism would permit the same steady-state net uptake of calcium by substrates of differing affinities.

When oxalate was present (see Table 29) the free intravesicular calcium concentration was kept low so that there was no feedback inhibition of substrate hydrolysis, over the time course of the experiment the calcium taken up in the different cases was a reflection of the rate of uptake in each case. Hence conditions with and without oxalate produced different results for the ratio of the amount of calcium taken up when ATP was the substrate to the amount of calcium taken up when other substrates were used. In MITO none of these alternative substrates were capable of supporting uptake. Neither succinate alone nor succinate plus ATP could increase the uptake by the MITO fraction from 1 or 0.3uM free calcium above the amount obtained in the presence of ATP alone.

b) Effect of drugs.

The lack of effect of 0.1uM of verapamil and propranolol on calcium uptake by PM, SR and MITO is in contrast to the effect seen by Balzer (1972) and Balzer,

Makinose and Hasselbach (1968). However, in contrast to Balzer, Makinose and Hasselbach (1968) and Balzer (1972) I used no oxalate, and a lower free calcium concentration (1uM) than did Balzer (1972) and Balzer, Makinose and Hasselbach (1968) (approximately 20uM). The inhibition produced by chlorpromazine was slightly higher in the experiments of Balzer, Makinose and Hasselbach (1968) than in mine. Just as Batra (1973b) showed chlorpromazine to be more potent at inhibiting calcium uptake by mitochondria than by sarcoplasmic reticulum isolated from frog skeletal muscle, so my results show it to be more potent in the MITO than in the PM or SR fractions from rat skeletal muscle. No increase in the amount of calcium taken up was seen when low concentrations of verapamil and chlorpromazine were used; however, if an increase in the rate of uptake did occur under these conditions it might have been masked in PM and SR by the leak of accumulated calcium. Drug effects in the presence of oxalate were not tested.

c) Effects of divalent ions and lanthanum.

Martonosi and Feretos (1964) found barium and strontium to be poor in competing with calcium for uptake by sarcoplasmic reticulum preparations, whilst Weber, Herz and Riess (1966) showed strontium to be taken up at a similar rate as calcium by sarcoplasmic reticulum,
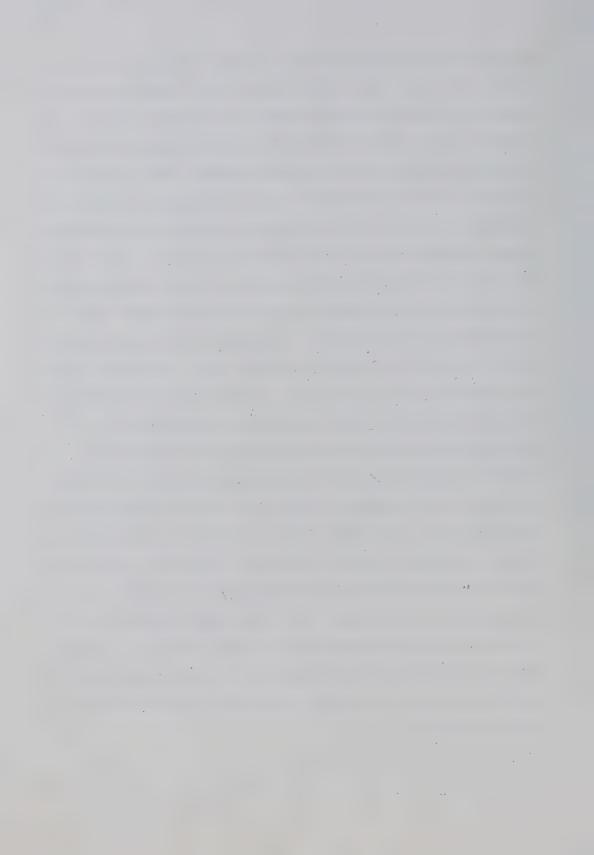
although the affinity of the sarcoplasmic reticulum was much greater for calcium than for strontium. In my experiments (Table 33) strontium clearly reduced the uptake of labelled calcium, whilst barium was without significant effect in all three fractions. Uptake of labelled calcium in the MITO fraction was reduced more effectively by strontium than by calcium itself, which might indicate that these mitochondria had a higher affinity for strontium than for calcium under these conditions. The effects of the addition of extra divalent cations on isotope dilution have been discussed in section A.3.d) of this chapter. By their responses to the different divalent ions PM and SR appear to have calcium uptake mechanism which differs from that of MITO. Table 34 shows that pre-incubation with high concentrations of lanthanum greatly reduce the ability of PM and SR to take up calcium; there were no differences in the responses of PM and SR to this ion.

4) Summary of work on rat skeletal muscle.

In summary, the calcium uptake demonstrated by the MITO fraction was obviously different from that of the PM and SR fractions. It showed a different time course, a different dependence on calcium concentration and on substrate; it also showed a different susceptibility to drugs and divalent ions, and a different rate from that of

PM or SR and was also blocked by azide when the others were not. On the other hand, very little difference except in amount of calcium taken up was seen between PM and SR. What justification is there then for saying that the plasma membrane of skeletal muscle does possess a calcium uptake mechanism? As was discussed earlier the amount of material derived from the sarcoplasmic reticulum which contaminates the PM fraction is unknown. The data in Tables 25 and 27 and in Figures 5 and 6 indicate that if all the calcium uptake in the PM fraction were due to contaminating sarcoplasmic reticulum, then approximately half the protein in the PM fraction must originate from the sarcoplasmic reticulum. However, the activities of the plasma membrane marker enzymes were enriched 3 to 4 fold in the PM fraction over those in the SR fraction. It clearly cannot be stated that calcium uptake by the plasma membrane of skeletal muscle has been unquestionably demonstrated. I conclude that a fraction enriched in plasma membrane markers takes up calcium by a mechanism which is in all respects tested similar to that of the sarcoplasmic reticulum. The fact that uptake in the PM fraction was potentiated more by oxalate than was uptake by the SR fraction could represent a greater leakiness of the PM vesicles or a greater ability of the SR vesicles to bind calcium.
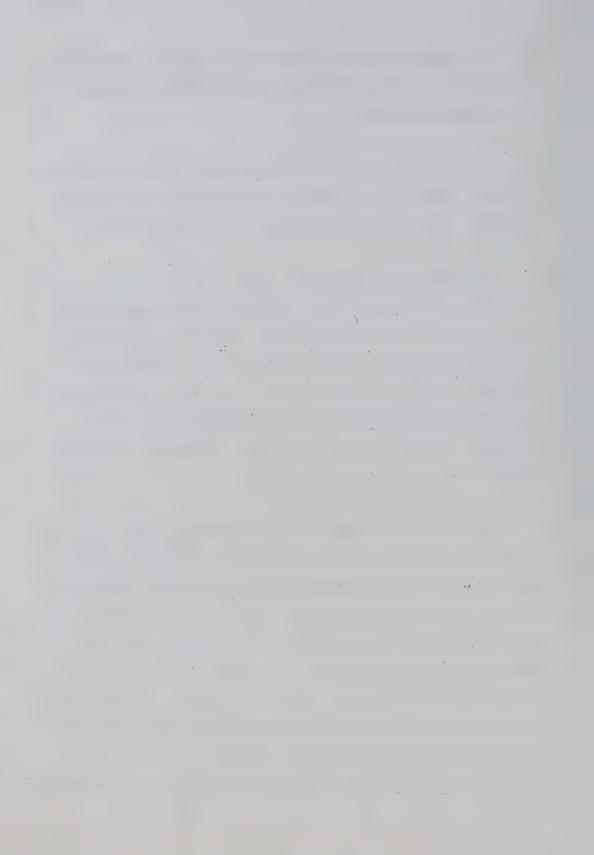
C. The similarities and differences between subcellular fractions of rat myometrium and subcellular fractions of rat skeletal muscle.

I will now summarize what has been accomplished and briefly compare and contrast the results obtained from skeletal muscle with those from uterine smooth muscle.
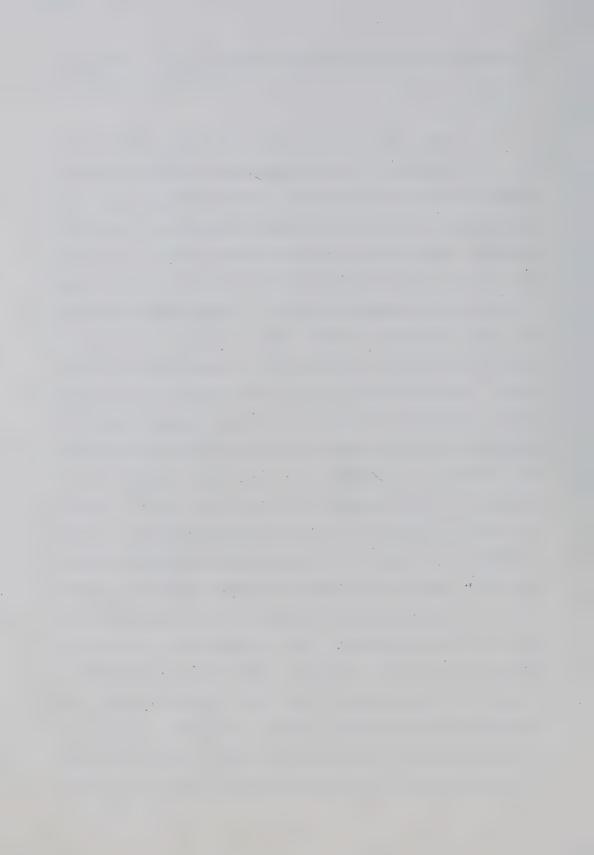
Mitochondrial fractions from both skeletal and uterine smooth muscles were isolated in reasonable purity. The calcium uptake properties of the two mitochondrial fractions were very similar, both in their affinities for calcium and in their rates. In both muscles the mitochondrial fraction had lower affinities and rates of calcium uptake than did the respective vesicular fractions.

The attempt to divide the microsomes from the two different muscles into fractions from particular cellular origins was more successful in the case of the myometrium than of skeletal muscle such that more confidence can be placed in the assignment of a calcium uptake mechanism to subcellular loci of the myometrium than of skeletal muscle. Thus plasma membrane, smooth endoplasmic reticulum and rough endoplasmic reticulum from myometrium can accumulate calcium with almost exactly similar properties whilst plasma membrane as well as sarcoplasmic

reticulum from skeletal muscle may be able to accumulate calcium.

The fact that calcium uptake properties were retained by the skeletal muscle membranes suggests that the technique for isolation did not severely damage the same properties in the smooth muscle membranes. However the skeletal muscle sarcoplasmic reticulum which I prepared was able to bind less calcium in the absence of oxalate than other sarcoplasmic reticulum preparations described in the literature, which might indicate some loss of calcium binding as a result of the isolation technique. There are some rather striking differences in the calcium uptake properties of the fractions isolated from the different muscles, which could conceivably be produced by the isolation procedure but are more likely to be inherent. Most striking of course is the rate of uptake; at 37ºC, the skeletal muscle SR fraction took up as much calcium in 30 seconds as the myometrial fraction did in 10 minutes, whilst in 20 seconds at 20ºC the skeletal muscle SR took up approximately 16 times more calcium than did the uterine fractions. Such differences in rates of calcium uptake are consistent with the differences in rates of relaxation of the two types of muscle. The skeletal muscle fractions showed themselves capable of taking up much more calcium at lower concentrations of calcium (almost 5 times more at 0.3uM, and more than 3

times more at 0.03uM) than did the smooth muscle fractions. Whether this difference is due to the fact that the myometrial contractile proteins are less sensitive to calcium than the skeletal ones over the range 0.03-0.3uM cannot be said until these properties have been directly investigated. Uptake by skeletal muscle fractions was greatly increased in the presence of oxalate, whereas that by smooth muscle ones was not; this indicates some differences and was discussed earlier. However, what these differences are cannot be decided from the results obtained here. The effect of several ions on caclium uptake revealed some similarities in the mechanism of uptake in skeletal and smooth muscle fractions, and the effects of drugs, in some cases where conditions used were the same, also showed similarities; in cases where drug studies were not repeated on the skeletal muscle fractions which I prepared, the effects seen on the smooth muscle fractions were similar to those seen by others on skeletal or cardiac muscle microsomes. A major difference was that uptake by the smooth muscle fractions could not be supported by a variety of substrates other than ATP whilst that by skeletal muscle fractions could. The extra-splitting of ATP due to added calcium was small and not always significant in the smooth muscle fractions.

Thus, despite its much lower rate of uptake, and its lower affinity for calcium, the smooth muscle calcium

uptake mechanism was in some ways very similar to that of skeletal muscle; however, the difference in substrate dependence indicates that it was not merely a ''diluted'' version of the skeletal muscle calcium uptake processes.

APPENDIX:

The association constants for the interaction between calcium and EGTA.

In a solution which contains a dissolved calcium salt and a dissolved EGTA salt the following equilibrium exists (amongst others):

$$Ca^{2+} + EGTA^{4-} = CaEGTA^{2-}$$

and the concentrations of the various species are related by K, the true association constant for $CaEGTA^{2-}$ such that

$$K = \frac{[CaEGTA^{2-}]}{[Ca^{2+}][EGTA^{4-}]} \qquad (1)$$

In normal experimental circumstances, however, several ions are present (e.g., H^+, K^+, Mg^{++} etc.) which may also interact with EGTA and/or calcium and thus influence the equilibrium of the Ca^{2+}, $EGTA^{4-}$ reaction. In this latter case the concentrations of the various species are related by K', the apparent association constant for $CaEGTA^{2-}$ such that

$$K' = \frac{[CaEGTA^{2-}]}{[Ca^{2+}]([EGTA]total - [CaEGTA^{2-}])} \qquad (2)$$

Where [EGTA] total is the concentration of all possible

forms of EGTA.

In the type of solutions used in this thesis a wide number of ionic species are present, various forms of ATP, H^+, K^+, Mg^{2+}, Cl^-, H.imidazole$^+$ etc. Many of these ions, but most notably H^+, Mg^{2+} and imidazole, can interact with various forms of EGTA (see Chaberek and Martell, 1959; Schwartzenbach et al., 1957; and Godt, 1974) and thus influence K'.

Thus a rigorous mathematical description of K' should take into account the effects of all ions present upon the amount of CaEGTA^{2-} that can be formed. Unfortunately, the nature of the interaction between imidazole and EGTA is not precisely known and there are as yet no constants to describe the interaction (Ogawa , 1968; Godt, 1974). The problem of the effect of imidazole on K' has usually been avoided by using values for K' determined in a medium containing imidazole (Ogawa, 1968; and Godt, 1974).

Similarly, the effects of all ions other than H^+ upon K' have normally been ignored in mathematical descriptions of K' in relation to K (see Ringbom, 1963; and Godt, 1974). Godt (1974) presents evidence from which he concludes that ATP, Mg^{2+} in concentrations normal for physiological experiments and K^+ over the range 50-300mM have little effect upon K'.

If calcium binds only to one form of EGTA, namely EGTA^{4-} (which is not strictly true though the affinity constant for CaHEGTA^{-1} is quite low, (Sillen and Martell, 1964)) and there is only one site of calcium-binding per molecule of EGTA (as is the case, Godt, 1974) and one accepts the negligible effect of ions other than H$^+$ on the value of K', then the following treatment is valid.
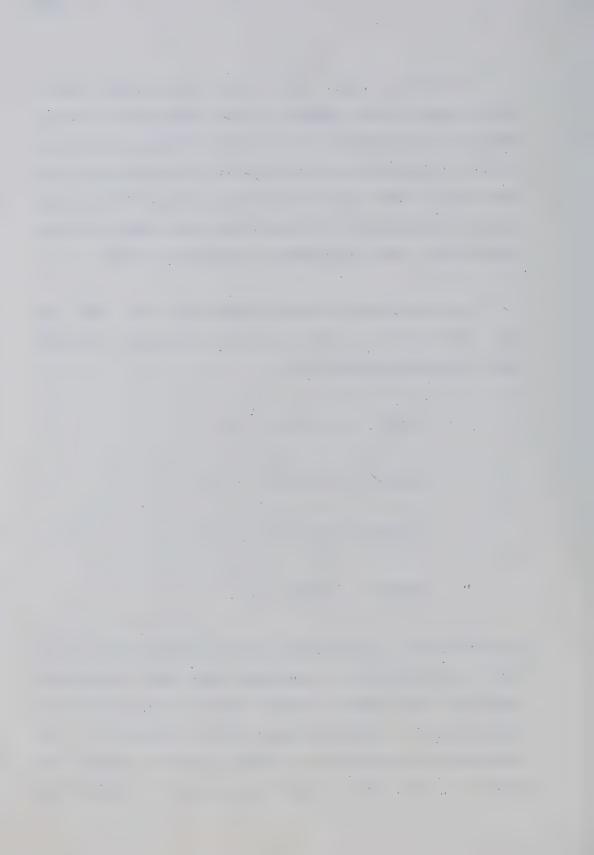
The interactions between hydrogen ion and EGTA are well understood and the following reactions can occur under appropriate conditions.

$$H_4 EGTA \rightleftharpoons H_3 EGTA^- + H^+$$

$$H_3 EGTA^- \rightleftharpoons H_2 EGTA^{2-} + H^+$$

$$H_2 EGTA^{2-} \rightleftharpoons HEGTA^{3-} + H^+$$

$$HEGTA^{3-} = EGTA^{4-} + H^+$$

The association constants for all the species given on the left in the above set of equations have been determined (Chaberek and Martell, 1959), thus the concentration of each species in a given solution can be calculated. The association constants for $H_4 EGTA$, $H_3 EGTA^-$, $H_2 EGTA^{2-}$ and $HEGTA^{3-}$ are 100, 479, 7.1×10^8 and 2.7×10^9 respectively

(Chaberek and Martell, 1959). At the pH values used in similar types of experiments to those in this thesis (normally in the range of pH6-8) it can be seen that the amounts of H_4EGTA and H_3EGTA^- present will be negligible, while those of H_2EGTA^{2-} and $HEGTA^{3-}$ will not. If KH_2EGTA is the association constant for H_2EGTA^{2-} and $KHEGTA$ is the association constant for $HEGTA^{3-}$ then

$$KH_2EGTA = \frac{[H_2EGTA^{2-}]}{[HEGTA^{3-}][H^+]} \tag{3}$$

and

$$KHEGTA = \frac{[HEGTA^{3-}]}{[EGTA^{4-}][H^+]} \tag{4}$$

Thus when Ca^{2+} and EGTA are together in solution at near physiological pH

$$[EGTA]total=[CaEGTA^{2-}]+[EGTA^{4-}]+[HEGTA^{3-}]+[H_2EGTA^{2-}] \tag{5}$$

Rearranging (3) gives

$$[H_2EGTA^{2-}] = KH_2EGTA \times [HEGTA^3][H^+] \tag{6}$$

and rearranging (4) gives

$$[HEGTA^{3-}] = KHEGTA \times [EGTA^{4-}][H^+] \tag{7}$$

and substituting for [HEGTA³⁻] in (6) gives

$$[H_2EGTA^{2-}] = KH_2EGTA \times KHEGTA \times [EGTA^{4-}] [H^+] [H^+] \qquad (8)$$

Substituting for [HEGTA³⁻] and [H₂EGTA²⁻] in (5) gives

$$[EGTA]total-[CaEGTA^{2-}] = [EGTA^{4-}] (1+KHEGTA \times [H^+]+$$
$$KHEGTA \times KH_2EGTA \times [H^+]^2)$$

$$(9)$$

by rearranging (2) and substituting in (9) we obtain

$$[CaEGTA^{2-}] = \frac{[EGTA^{4-}] (1+KHEGTAx[H^+]+KHEGTAxKH_2EGTAx[H^+]^2)}{K'x[Ca^{2+}]}$$

$$(10)$$

by substituting (1) in (10) we obtain

$$K = K' (1+KHEGTA \times [H^+] + KHEGTA \times KH_2EGTA \times [H^+]^2)$$

Thus the true and apparent association constants for CaEGTA²⁻ are expressed in terms of values which have been determined or can be determined. It can be seen that small changes in the value of [H⁺] will greatly influence the value of K'; thus the neccessity for rigid control of pH during experimental work in order to maintain the required free calcium concentration.

The more correct approach whereby the effect of every ion in solution is taken into consideration would be additionaly complex. However, it can be seen from the simplified correction used here how small changes in operating conditions can effect K' and thus the free calcium concentration. Until all the influences on the formation of $CaEGTA^{2-}$ are known and included in the equation (e.g., imidazole), the variations in K' will not be exactly understood.

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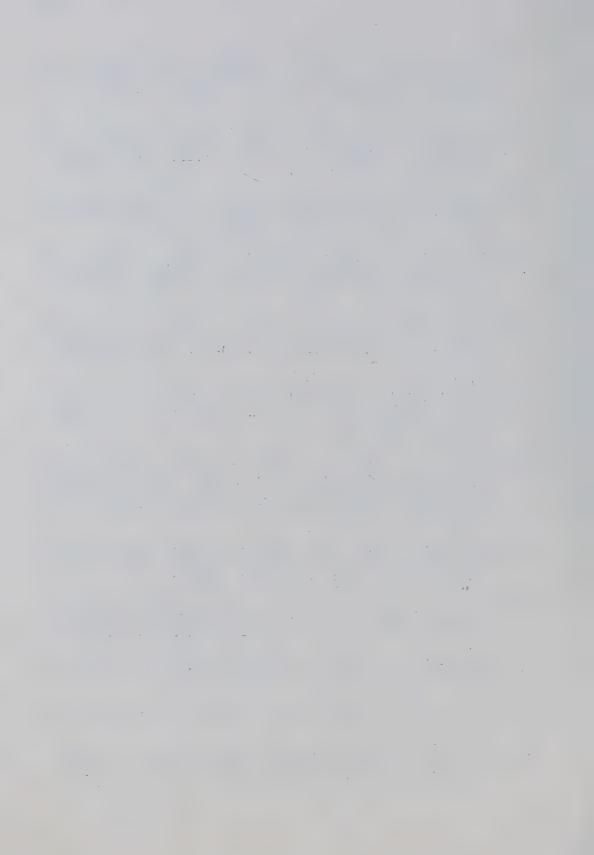
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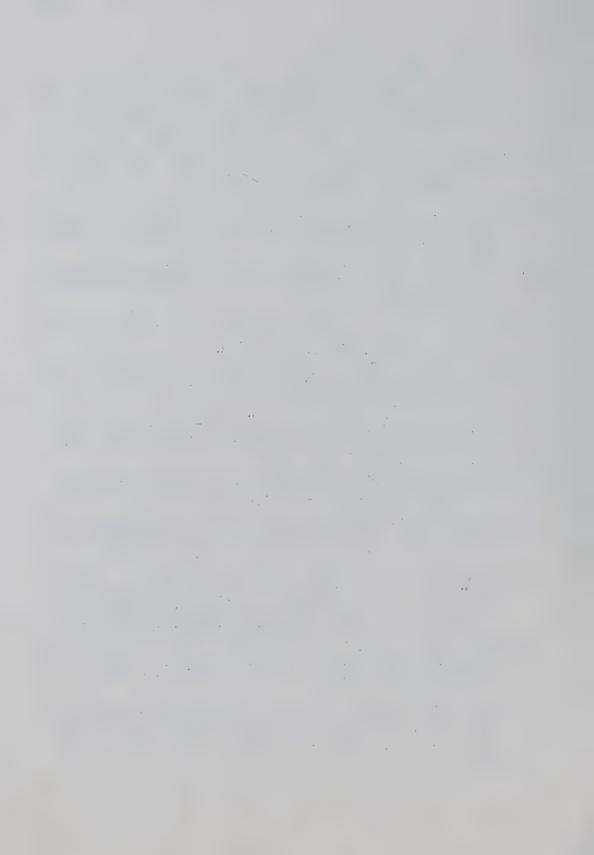Bianchi, C.P., and T.C. Bolton. 1967. Action of local anesthics on coupling systems in muscle. J. Pharm.

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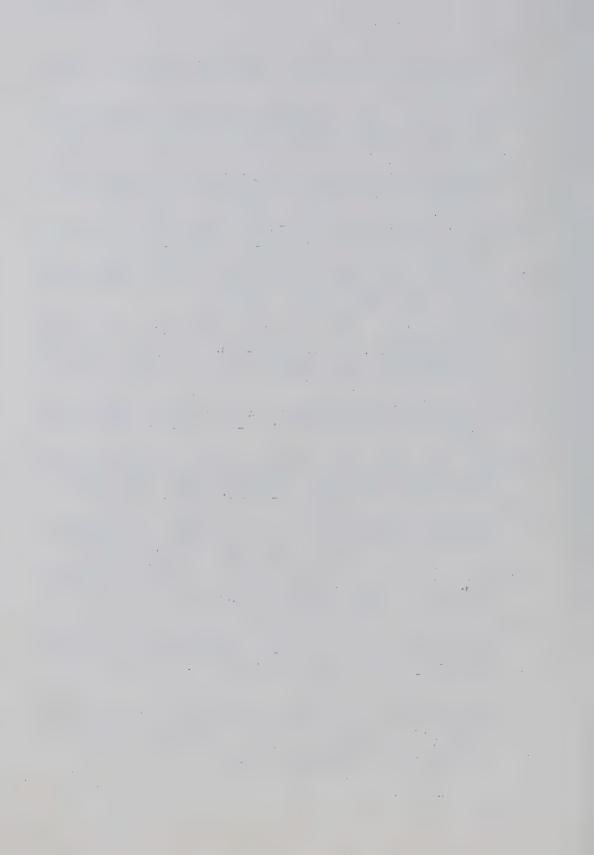
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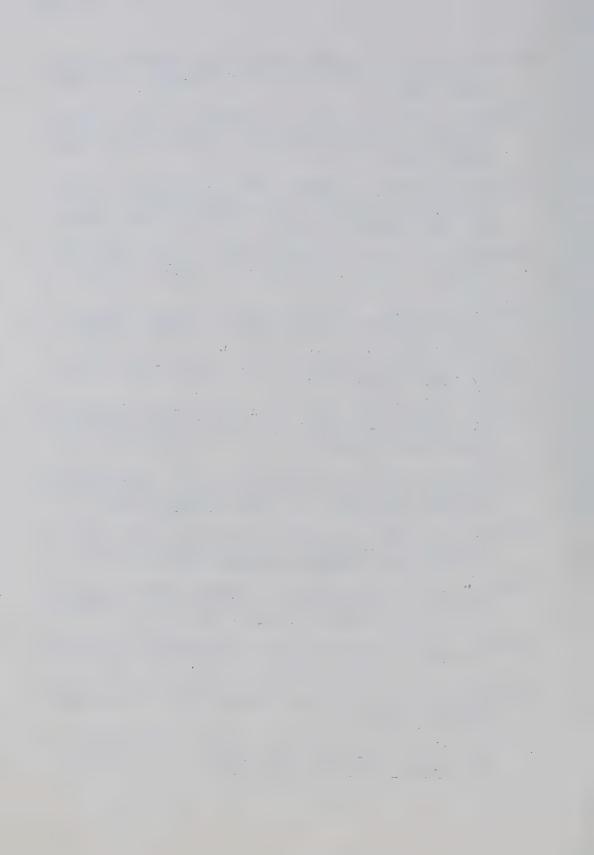DeLuca, H.F., and G.W. Engstrom. 1961. Calcium uptake by rat kidney mitochondria. Proc. Natl. Acad. Sci. 47:1744.

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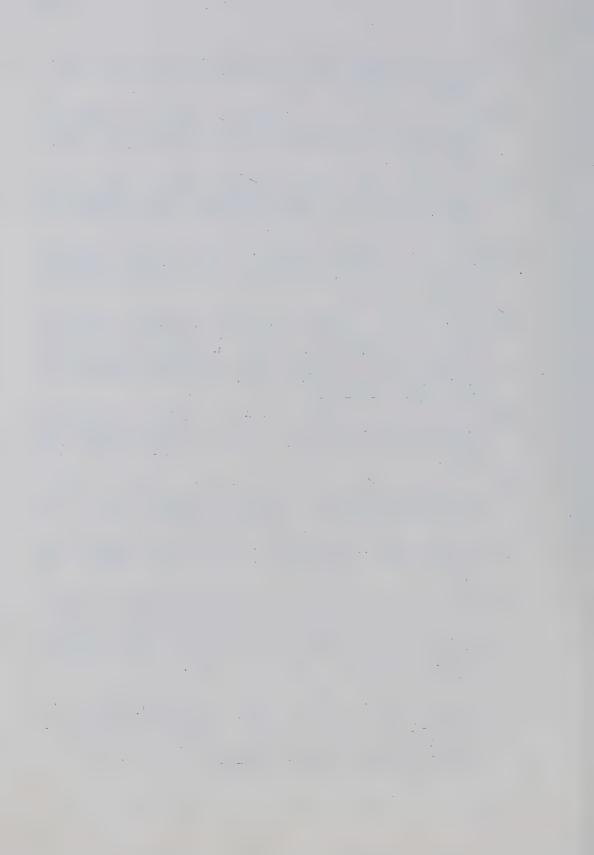
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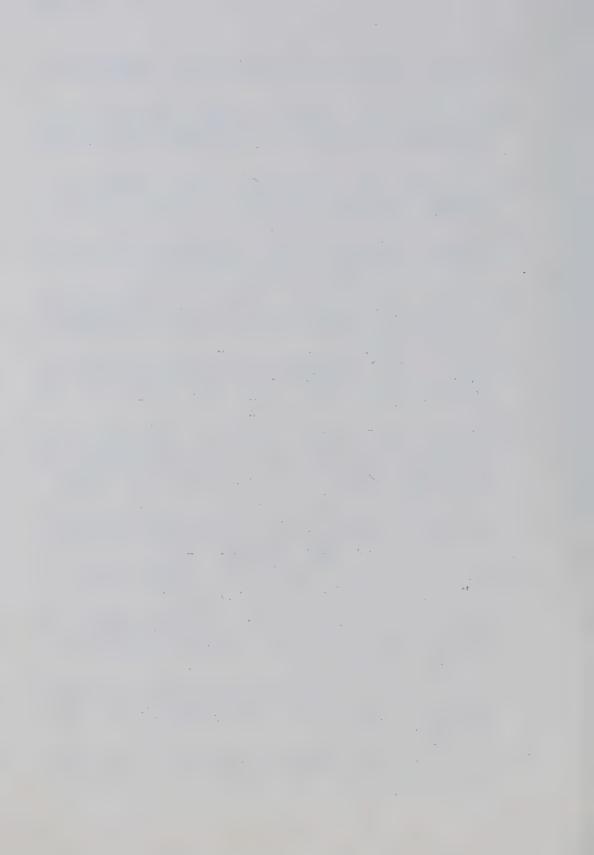
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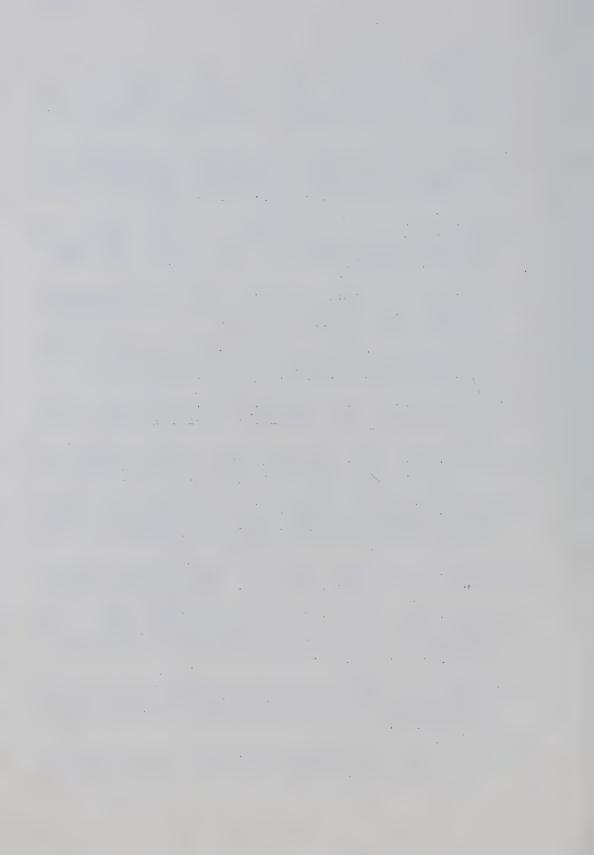
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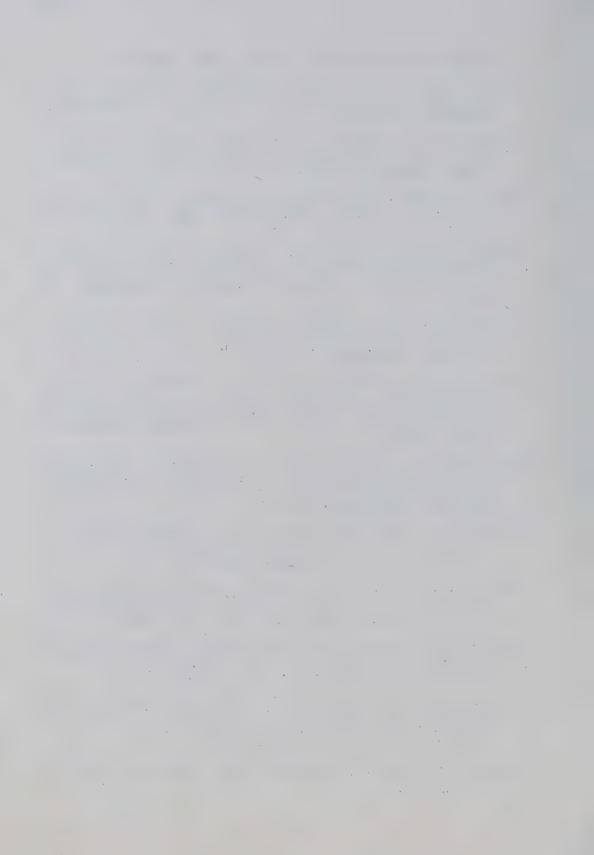Goodford, P.J. 1967. The calcium content of the smooth muscle of the guinea-pig taenia coli. J. Physiol.

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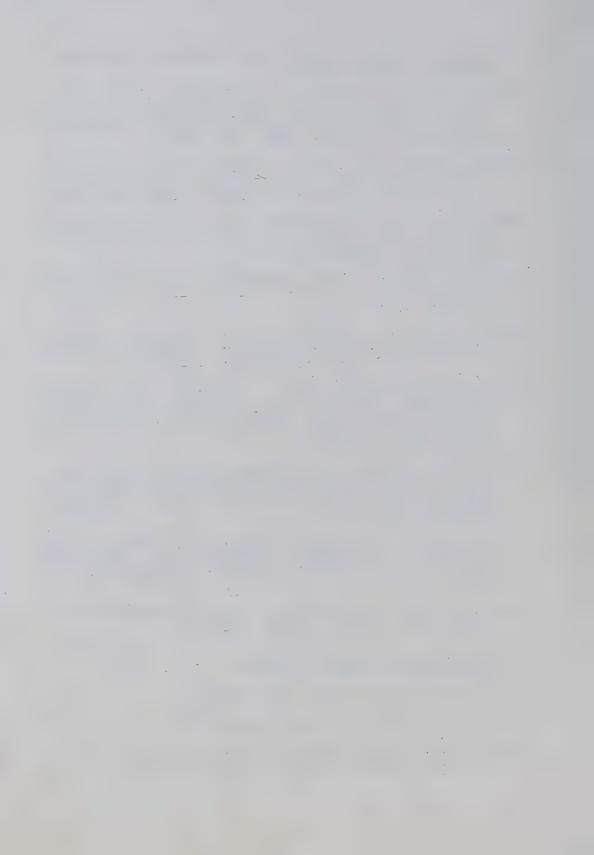
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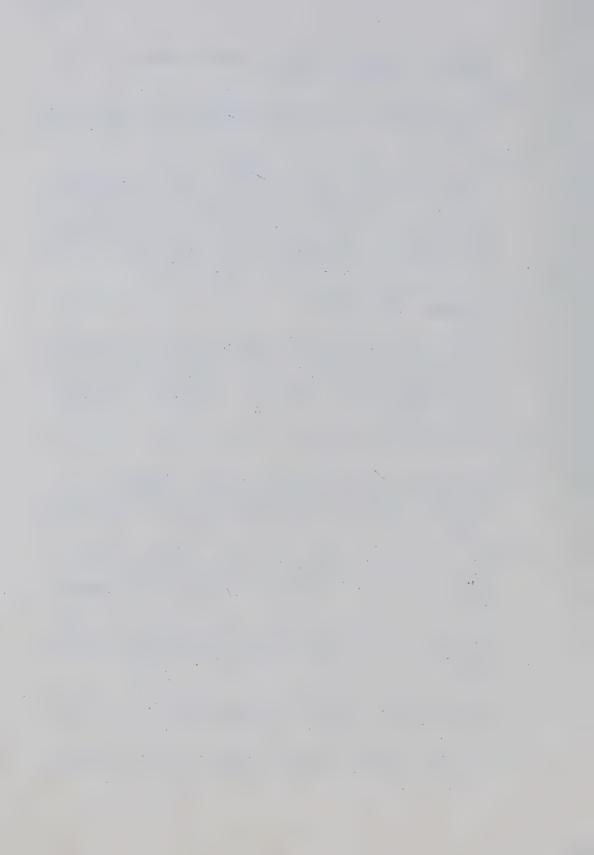
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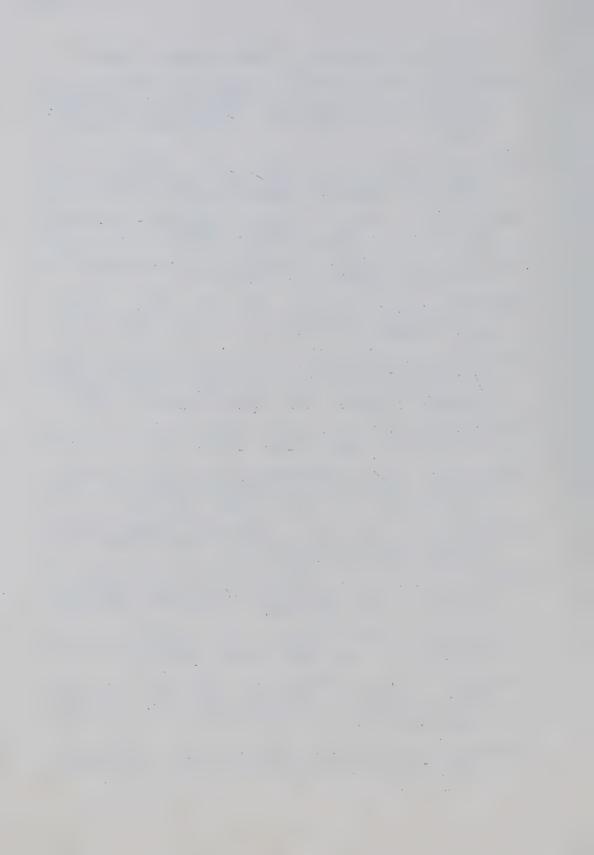Lehninger, A.L., E. Carafoli, and C.S.   Rossi.   1967.
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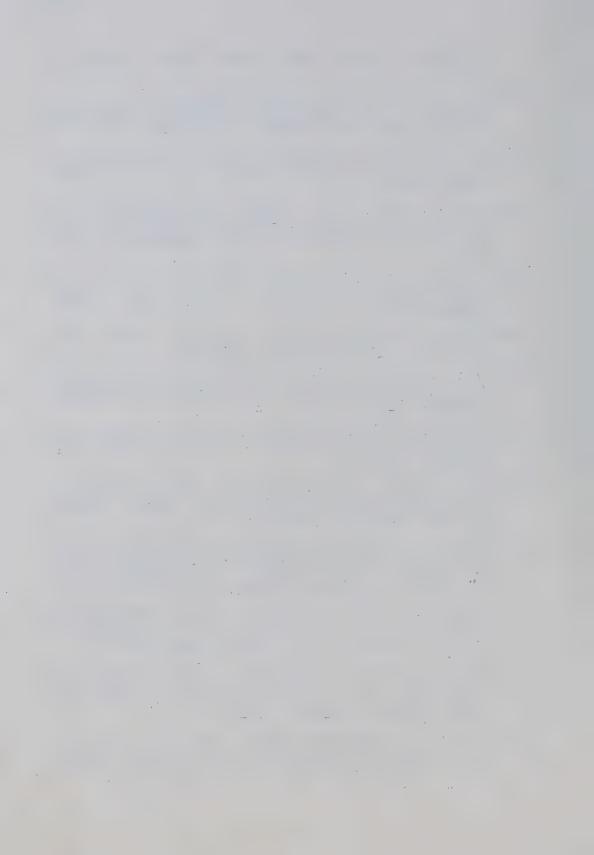   Biochem. Biophys. Res. Commun. 10:444.

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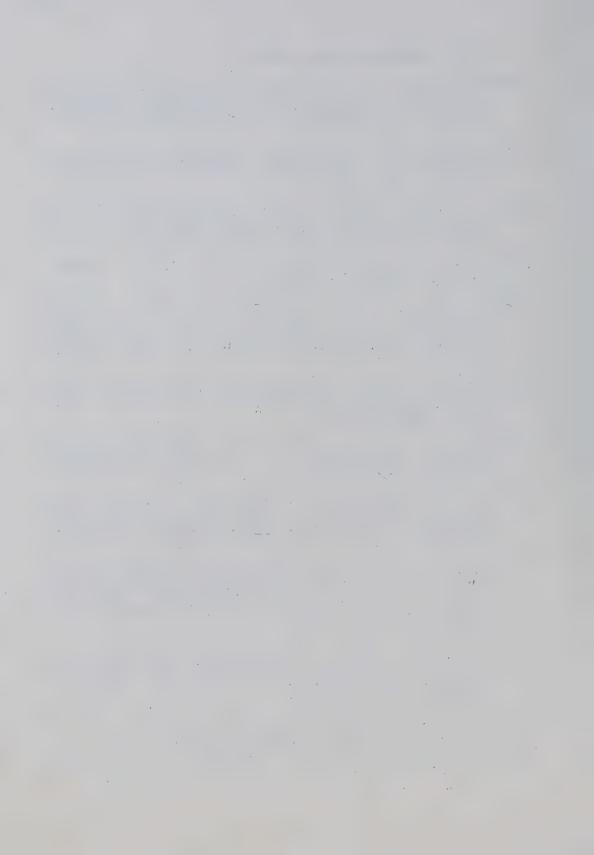in the smooth muscle cells of the guinea-pig taenia

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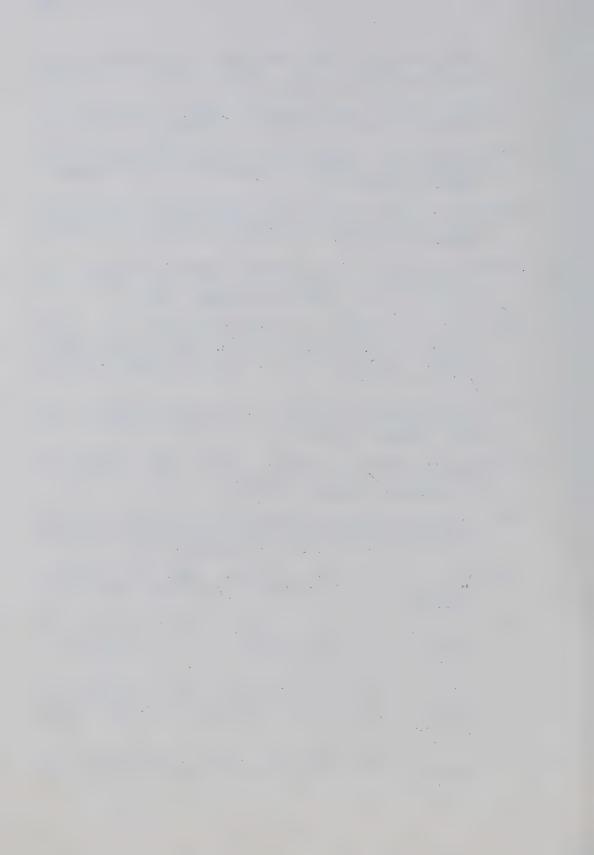Mueller, H., and S.V. Perry. 1961. The chromatography of the meromyosins on diethylaminocellulose. <u>Biochem.</u> <u>J.</u> <u>80</u>:217.

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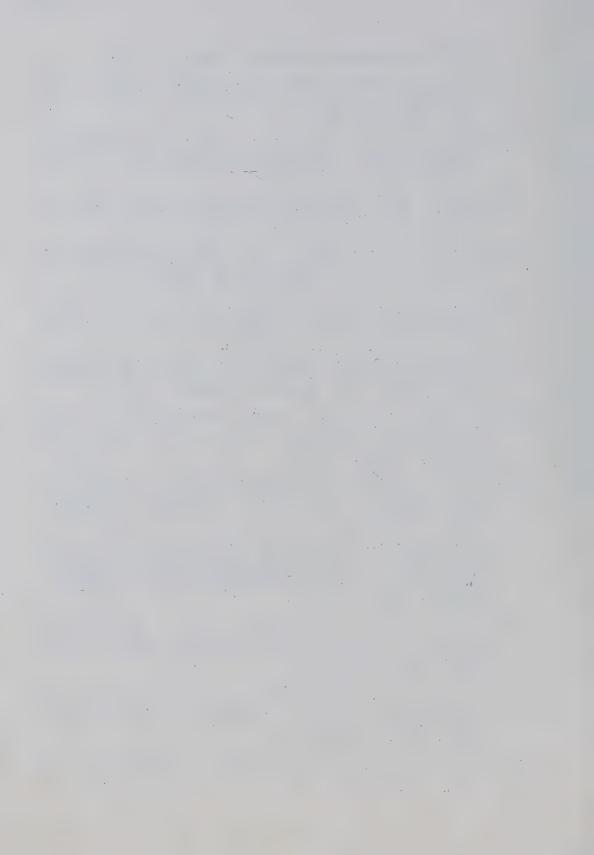
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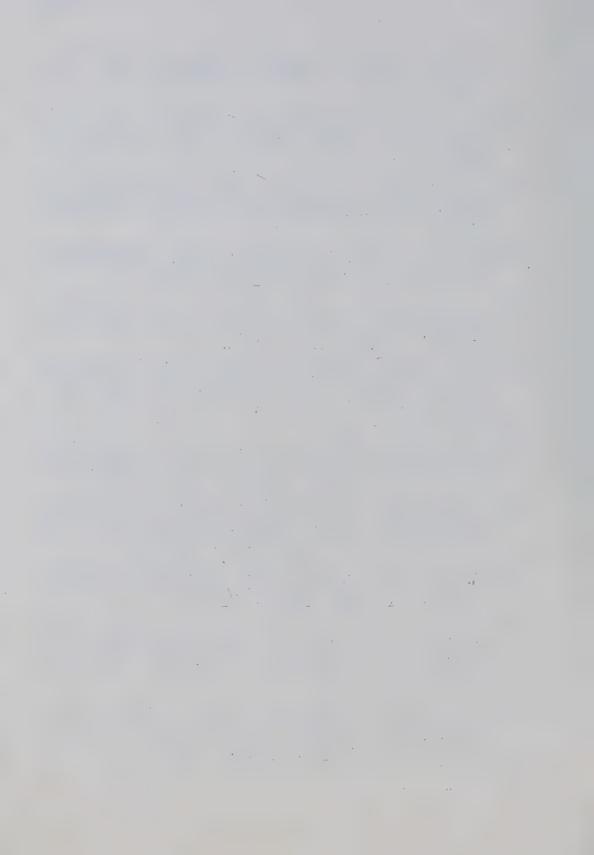
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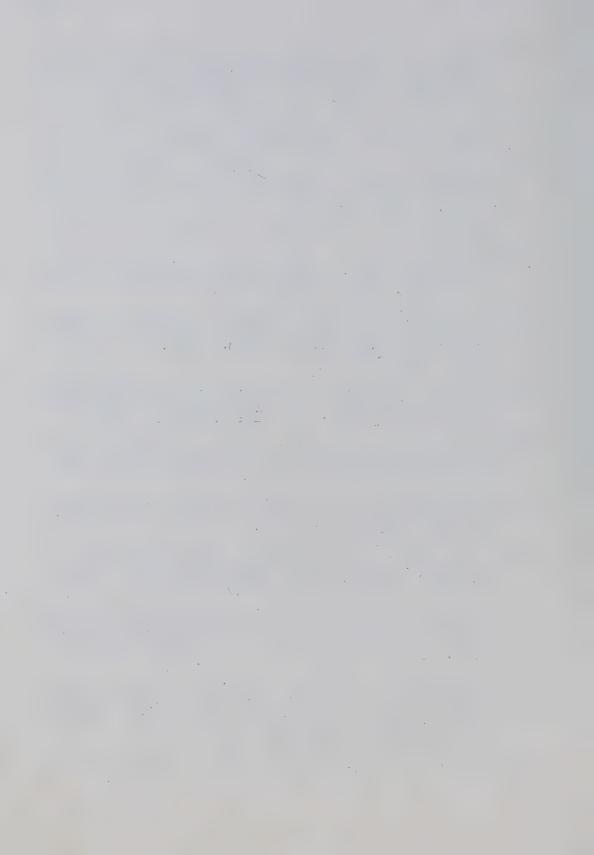
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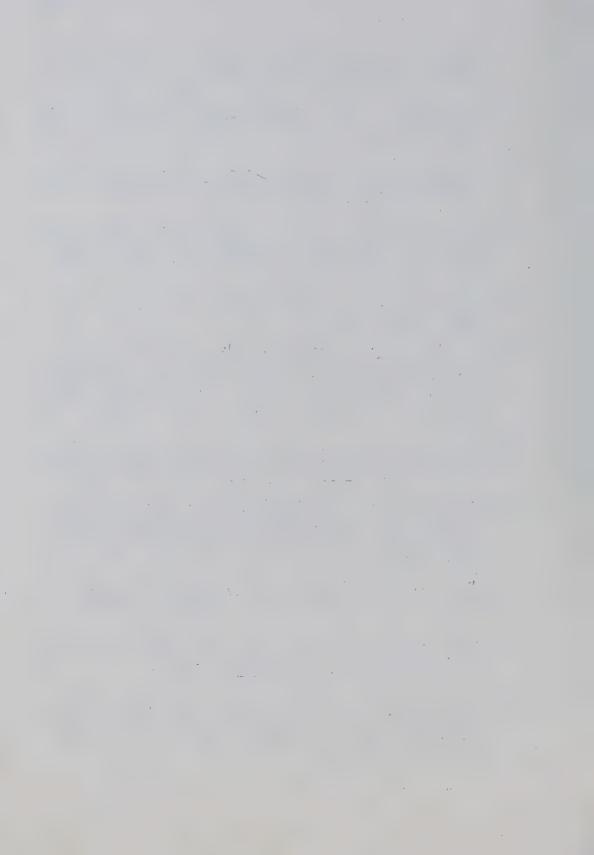Sitrin, M.D., and D.F. Bohr. 1971. Ca and Na interaction in vascular smooth muscle contraction. Am. J. Physiol. 220:1124.

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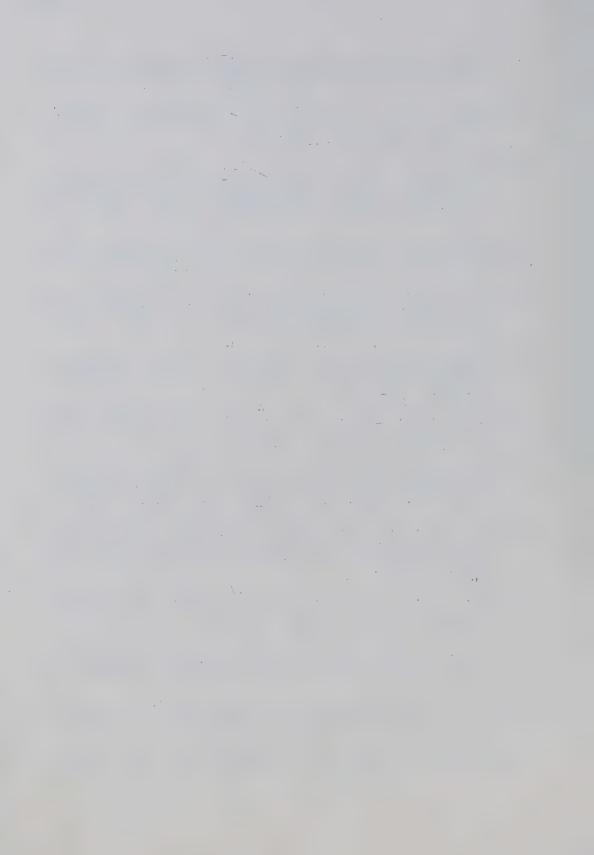
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